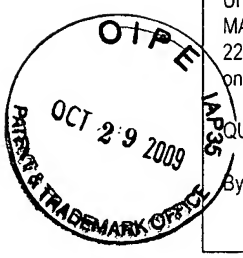


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QUINE INTELLECTUAL PROPERTY LAW GROUP, P.C.
By: Kimberly Cheung
Kimberly Cheung

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|---|---|---|
| Appl. No. | : | 10/044,463 Confirmation No. 9878 |
| Applicant | : | Davide R. Grassetti, et al. |
| Filed | : | January 10, 2002 |
| TC/A.U. | : | 1617 |
| Examiner | : | Shengjun Wang |
| Docket No. | : | 107-000110US |
| Customer No.: | | 22798 |

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**APPEAL BRIEF**

REAL PARTY IN INTEREST

The real part of interest in the present appeal is Grassetti Family Trust, the assignee of the above-referenced application.

RELATED APPEALS AND INTERFERENCES

Appellant, Appellant's Attorney, and the assignee of the above-referenced application are unaware of any appeals or interferences that will

directly affect, be directly affected by, or have a bearing on, the Board's decision in the present appeal.

STATUS OF CLAIMS

On March 11, 2009, Appellant appealed from the final rejection of claims 1 to 3, 5 to 16 and 20 to 24. The Office has replied with the new non-final Office Action of August 17, 2009; to which Appellant have elected not to respond. As originally filed, the case included claims 1 to 24. In the Restriction Action of June 15, 2005, Appellant was requested to elect species. Based on the species election of July 3, 2005, the Office withdrew claims 3-4, 7-9, and 13-16 from current consideration, as being drawn to non-elected species. Claims 4, and 17 to 19 were cancelled in Appellant's Request for Continued Examination (RCE) of August 9, 2006. Accordingly, Appellants believe that claims 1, 2, 5, 6, 10 to 12, and 20 to 24 are under consideration.

STATEMENT OF AMENDMENTS

No Response was filed in response to the new non-final Office Action of August 17, 2009, provided by the Office in reply to Applicant's Appeal Brief of April 29, 2009. The claims were not amended in response to the final Office Action mailed November 13, 2008. Accordingly, the appealed claims are the claims as provided in the Amendments of the RCE dated

February 12, 2008, as filed in response to the Panel Decision from Pre-Appeal Review dated January 14, 2008.

## SUMMARY OF CLAIMED SUBJECT MATTER

Appellants' invention provides methods of modulating an immune response by identifying an individual in need of immune response modulation and administering effective amounts of thione-forming disulfide (TFD) compounds of certain structure to the individual in need of immune response modulation, wherein the individual in need is other than an individual infected with a retrovirus. Support for the claims can be found throughout the specification, e.g., in the Examples section starting at paragraph 129. Specifically, support for identifying an individual in need of immune response modulation and administration of TFDs can be found at paragraphs 33, 43, 108, 111 and in the section entitled "Immunomodulation in Treatment of Diseases and other Ailments" starting at paragraph 113, and in the section entitled "Administration of Thione-Forming Disulfides starting at paragraph 93. Support for The TFD structures of the claims can be found, e.g., at paragraph 67.

The appealed claims are set forth in Appendix A.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

In the new non-final Office Action dated August 17, 2009, all claims under consideration, including claims 1, 2, 5, 6, 10 to 12, and 20 to 24, were rejected under title 35 § 102(b) based on alleged anticipation by Grassetti et al., U.S. patent 5,662,364. In addition, all claims under consideration, including claims 1, 2, 5, 6, 10 to 12, and 20 to 24, were rejected under title 35 § 103(a) for alleged obviousness based on Grassetti et al., U.S. patent 5,662,364, in view of Oliver (Cancer Surveys, 13:173-204, 1992) and Tagawa (Current Pharm. Design 6: 681, 2000).

ARGUMENTS

**Rejection of claims 1, 2, 5, 6, 10 to 12, and 20 to 24 under 35 U.S.C. §102(b).**

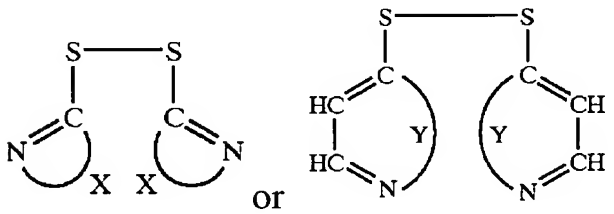
Claims 1, 2, 5, 6, 10 to 12, and 20 to 24 were rejected under 35 U.S.C. §102(b) as allegedly anticipated by Grassetti (U.S. 4,378,364), as evidenced by Oliver (Cancer Surveys, 13:173-204, 1992) and Tagawa (Current Pharm. Design 6:681 (2000)). These claims are not anticipated and stand or fall together.

As a preliminary matter, Appellants note that the present rejections are essentially the same rejections for alleged anticipation argued in the Appeal

Brief of April 29, 2009, (never replied to by the Office). Appellants again note that, even in light of the new Oliver reference, Grassetti '364 does not teach all limitations of the claims. Appellants again note that the cited evidence of record actually supports the fact that not all cancer patients are in need of immune response modulation. Therefore, administration of a TFD to a cancer patient by Grassetti '364 (to enhance their feeling of well-being) does not inherently identify and administer to an individual in need of immune response modulation.

Rejected independent claim 1 is as follows:

> 1. (Previously presented) A method for modulating an immune response comprising:
>
> identifying an individual in need of immune response modulation;
>
> administering to the individual in need of immune response modulation an effective amount of a thione-forming disulfide comprising



wherein X and Y represent atoms necessary to form a five-membered or six-membered substituted or unsubstituted heterocyclic ring;

wherein the immune response is selected from the group consisting of: a cellular response, a humoral response and an innate immune response; and,

wherein the individual is other than an individual infected with a retrovirus;

thereby modulating the immune response.

The rejections are inadequate because the Examiner has failed to state a *prima facie* case. Moreover, the rejections of these claims are inappropriate because the cited reference does not explicitly or inherently teach at least the limitations of methods comprising: 1) identifying an individual in need of immune response modulation, and 2) administering a thione-forming disulfide (TFD) to an individual in need of immune response modulation.

In order for a reference to anticipate an invention, the reference must teach each and every element of the claimed invention. That is, in order for a reference to anticipate an invention, "all limitations of the claim are found in the reference, or 'fully met' by it." *Kalman v. Kimberly-Clark Corp.*, 218 USPQ 781, 789 (Fed. Cir. 1983).

Grassetti '364 (same deceased inventor of the present application) administered thione-forming disulfides (TFDs) to cancer patients to improve their "general physical condition" and improve "their feeling of well-being". See, e.g., column 4, line 61. The current rejections require that this administration of TFDs both inherently identified and inherently treated an individual in need of immune response modulation. However, the Office's own references of record clearly show that not all such patients are necessarily in need of immune response modulation.

The Examiner failed to reply to Appellant's Appeal Brief of April 29, 2009, apparently because abundant evidence of record clearly shows that not all cancer patients are inherently (necessarily) in need of immune response modulation. The new non-final Office Action in response to Applicant's Appeal Brief only adds the new Oliver reference that suggests in another context that cancer therapies may suppress immune responses to some degree. However, the cited cursory unsupported statement in Oliver does not expressly require of that all cancer patients are in need of immune response modulation, and does not does not change the bulk of more authoritative references of record that contradict the Examiner's conclusions.

**Grassetti '364 does not teach at least identifying an individual in need of immune response modulation.** This is a limitation in all the rejected claims, particularly independent claims 1 and 23.

At section 4 of the new non-final Office Action of August 19, 2009 (the Action), the rejection is based on the statement that the method step of:

> "'identifying an individual in need of immune response modulation;' is [allegedly] inherently met by the method of treating cancer patient disclosed in the reference, as [Appellants note that in previous Action the word "all" was used here, but has been removed in the present Action] cancer patients ... are recognized as 'in need of immune response modulation' See, particularly, the abstract in Tagawa and page 198 in Oliver. Oliver specifically states: 'All modalities of cancer therapy except hormone therapy (i.e., surgery, radiotherapy and chemotherapy) suppress immune responses." Emphasis added.

Controlling case law requires that for an aspect to be inherent in prior art, the aspect must necessarily be present in all embodiments. See, e.g., *In re Best*, 195 USPQ 430; *Ex parte Levy*, 17 USPQ2d 1461; and, *Continental Can Co. USA v. Monsanto Co.*, 20 USPQ2d 1746. For example, according to *Continental Can* missing descriptive matter must necessarily be present in the thing described in the reference.

The Examiner acknowledges that Grassetti '364 does not actually identify an individual in need of immune response modulation. Grassetti '364 had various patients with various conditions. The individuals were treated with a TFD to improve their feeling of well-being. For example, Grassetti '364 treated mice, healthy individuals, and cancer patients with a TFD (6,6'-dithiodinicotinic acid). Grassetti '364 did not particularly "identify" cancer patients, as distinguished over his non-cancer subjects. Further, although some individuals in the treated population had experienced chemotherapy, even assuming all members of this subgroup were immune suppressed to some extent, they were not particularly identified. Further, immune suppression is a matter of degree, and it is not clear any individual that may theoretically been immune suppressed was necessarily suppressed to a degree that they were in need immune response modulation.

Because chemotherapy-treated cancer patients are not particularly identified in Grassetti '364, and because not all those individuals exposed to chemotherapy are necessarily immune suppressed to a degree making them in need of immune response modulation, it can not be said that Grassetti '364 actually identified any individual in need of immune response modulation. Appellants note that the Examiner has never actually identified which

individual in Grassetti '364 was allegedly identified and in need of TFD administration for immune response modulation.

**Grassetti '364 does not inherently administer a TFD to any individual in need of immune response modulation.** It is not clear what the Examiner considers to be the key argument for the rejection. However, a shot-gun blast of diverse unrelated comments are present at pages 3 and 4 of the Action. Taken together, the unrelated arguments for alleged anticipation in the Action may be confusing, but they do not actually make the required showing of facts supporting the assertion that all cancer patients are inherently in need of immune response modulation.

**I.** In sections 1 and 4 of the Action, the Examiner suggests immunocompromised patients are individuals inherently in need of immune response modulation. However, the Examiner's conclusions in light of Oliver do not logically follow. For example, even assuming an individual in Grassetti is immune suppressed to some degree, according to Oliver, the unidentified individual is not necessarily immunocompromised so as to be in need of immune response modulation.

The Examiner argues that immune compromised individuals "can be" treated with TFDs and "may have lower than normal numbers of immune

cells", as suggested at paragraph 100 of the present specification. However, it does not necessarily follow that all immune compromised individuals are necessarily in need of immune response modulation. As evidence of such a necessity, the Examiner, at page 4, section 4, offers Oliver (Cancer Surveys, 13:173-204, 1992), stating "All modalities of cancer therapy except hormone therapy (i.e., surgery, radiotherapy and chemotherapy) suppress immune responses." However, even assuming all cancer patients are immune suppressed (and they actually are not), not all immune suppressed individuals and cancer patients are suppressed to a point of being compromised or in need of immune response modulation. Therefore, even assuming the Examiner is right that a Grassetti '364 patient was immune suppressed, the Examiner has not met the burden of showing common knowledge (MPEP 2144.03) that all cancer patients are in need of immune response modulation.

Assuming Oliver is correct in stating all "modalities of cancer therapy ... suppress immune responses", this does not necessitate a need for immune modulation. For example, suppression is a matter of degree. Is a mildly suppressed patient always in need of immune modulation? Is a successfully chemotherapy treated individual in need of immune response modulation?

The Examiner further cites the abstract of Tagawa as evidence that all cancer patients are in need of immune modulation. Tagawa, in the cited abstract, actually states that modulation of immune responses "is one of the strategies for cancer therapy. ... However, cytokines may induce toxic reactions or produce no substantial effects ..." Tagawa also notes that cancers can result from immunological disorders or defects of a host immunosurveilance system, but this acknowledges the fact that most cancers arise in individuals without such defects. In Figure 1, Tagawa shows how an unmodulated immune system normally works with, e.g., natural antigen presenting cells (APCs) activating cytotoxic T-lymphocytes (CTLs) to provide a normal CTL-mediated unmodulated immune response against a tumor *in vivo*. This is a clear demonstration that immune modulation is not necessarily needed by all cancer patients. The cited references teach that a need for immune response modulation is not inherent in individuals with a cancer.

In the Action of May 18, 2007, the Examiner also cited Barber et al., (U.S. 5,662,896) as support for the notion that all cancer patients are in need of immune response modulation. Because this reference contradicts the examiner's arguments, he no longer cites Barber. Barber, at the previously cited columns 1 and 2, makes it clear that cancer patients are not necessarily in need of immune modulation. For example, at column 1, line 35, Barber suggests that

30% of patients treated with surgery alone will have no recurrence. These cancer patents were not treated with immune modulators, and did not need them. Chemotherapies and radiation therapies also have success without resort to immunomodulation, even assuming they may immunosuppress to some degree. Barber actually avoids the use of immune modulators in his treatment for cancer patients. For example, in the paragraph traversing columns 2 and 3, Barber identifies toxins or antisense technology for direct administration to a tumor without immune modulators. The evidence goes on to clearly demonstrate the Examiner is incorrect in asserting that all cancer patients are inherently in need of immune modulation.

The cursory statement of Oliver does not require, or even suggest, that a treated cancer patient is in need of immune response modulation. The extensive fact based research and scientific review of Tagawa and Barber make it clear that cancer patients are not inherently in need of immune response modulation. Therefore, even though Grassetti '364 administered TFDs to treated cancer patients and others to improve their feeling of well-being, he did not necessarily (inherently) identify or administer the TFDs to an individual in need of immune response modulation.

**II.** In section 3 of the Action, the Examiner acknowledges that the Grassetti '364 treats and monitors individuals for effects on their feeling of well-being. Then, in an illogical attempt to characterize the treatments (expressly directed to treating well-being) as immune modulation treatments, the Examiner notes that Grassetti '364 suggests "[t]reatment with the regent should be continued for an adequate period of time both before and following surgery until the *natural defenses* of the organism have destroyed the remaining circulating cancer cells." Column 12, line 4; emphasis added by Examiner.

The Examiner's reference to Grassetti '364 column 12, line 4, is irrelevant. Continued well-being treatment with the TDFs before and following surgery until the natural defenses of the organism have destroyed the remaining circulating cancer cells does not teach identifying individuals in need of immune modulation or teach treatment by immune response modulation. It is not clear why this statement was presented in the Action. The statement actually teaches that natural (unmodulated) defenses are adequate to destroy the cancer cells, e.g., while the general physical condition and feeling of well-being is being enhanced. It is notable that at the time of the Grassetti '364 application filing, the dithiodinicotinic acid mode of action was thought to correlate to its close similarity to nicotine, niacin and serotonin, each of which can be mood elevators. There was no suggestion at the time of filing that the TFDs were

effective in modulating immune responses. In the context of the time (i.e., without hindsight) the statement suggests the patient's well being and general physical condition (see column 4, line 62 of Grassetti '364) should be maintained while the natural defenses destroy cancer cells without any suggestion of immune modulation (as further evidenced by Tagawa and Barber, discussed above).

**III.** At the bottom of page 3 in the Action, the Examiner comments the "preamble is generally not accorded any patentable weight." The Examiner's reference to patentable weight of the preamble appears to be irrelevant and immaterial to the issue of anticipation. The Examiner had previously noted in the Action of November 2, 2006, that "modulating an immune response" in the preamble was to be given no weight. In the Response of February 7, 2007, Appellant amended the claim to insert "thereby modulating the immune response" into the body of the claim, where it must be accorded weight. It is inexplicable that this issue of the preamble has continued to arise in succeeding Actions.

**IV.** Finally, in section 5 at page 4 of the Action, the Examiner quotes *In re Swinehart* for the proposition that recitation of a newly discovered property does not distinguish a composition over the same composition in the prior art.

This reference to *In re Swinehart* has also continued to arise in various Actions, without acknowledging and addressing the substance of previous Appellant rebuttals. For example, in the Appeal Brief of April 29, 2009, Appellant noted "[w]ith regard to section 5 of the Action, concerning *In re Swinehart* and inherent properties of compositions, Appellants direct the Board of Appeals to remarks of Appellants' February 7, 2007, Response, at page 8. For example, *Swinehart* is not on point because the rejected method claims are distinguished over the prior art by more than a mere inherent function of compositions in the prior art. That is, the present claims are not limited only to, e.g., a composition of CPDS but are methods claims with additional limitations." Of course, it is not novel to claim an old composition by merely reciting a new function. However, the current methods claims do not claim an old composition, but a new method of using a composition; as is appropriate, e.g., according to 35§101. Therefore, the repeated citation of *In re Swinehart* is inappropriate and not on point.

Because Grassetti '364 does not actually or inherently disclose the limitations of identifying an individual in need of immune response modulation, and administering a thione-forming disulfide (TFD) to an individual in need of immune response modulation, it can not be considered to anticipate any claim.

**Rejection of claims 1, 2, 5, 6, 10 to 12, and 20 to 24 under 35 U.S.C. §103(a).**

Claims 1, 2, 5, 6, 10 to 12, and 20 to 24 were rejected under 35 U.S.C. §103(a) as allegedly obvious based on Grassetti (U.S. 4,378,364), in light of Oliver (Cancer Surveys, 13:173-204, 1992) and Tagawa (Current Pharm. Design 6:681, 2000). These claims are not obvious and stand or fall together.

In the many Actions and years of prosecution, the Examiner has never raised an obviousness issue, until now. This is because an allegedly inherent but unknown activity of a composition does not render methods obvious for using that previously unknown activity. The obviousness rejections newly presented in the Action fail because the cited Grassetti '364 statement (column 12, line 4) does not teach TFDs can be used for modulation of immune responses.

A proper analysis under the recently reaffirmed *Graham v John Deere* standard demonstrates the non-obviousness of the invention. According to the Supreme Court in *KSR International Co v. Teleflex* (550 U.S. 398 (2007); 127 S. Ct. 1727, 1740-41, 82 USPQ2d 1385-1396 (US 2007)), the appropriate standard for analyzing questions of obviousness is that:

> the scope and content of the prior art are determined, differences between the prior art and the claims at issue are analyzed and the level of ordinary skill in the pertinent art is

> resolved. Against this background the obviousness or non-obviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unresolved needs, failure of others, etc. might be utilized to give light to the circumstances surrounding the origin of the subject matter to be patented.

*Id.* quoting *Graham v. John Deere of Kansas City* 383 U.S. 1, 17-18.

The current Examination Guidelines (e.g., MPEP 2143) and *KSR* require the Office in an obviousness rejection to provide a statement as to why one of skill would have combined known elements. Further, an obviousness rejection must include fact-based findings demonstrating: 1) a combination of reference elements describing each limitation of the claims, 2) known elements that function in the same way in the combination as in the references themselves, 3) the elements are combined by known methods, 4) the result of the suggested combination of elements would have been predictable, and 5) one of skill in the art would have expected success in providing the claim in light of the references. Here, the rejection fails each of these requirements, as applied to the *Graham* factors. Further, *KSR* requires that the Office should "identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does."

**All Limitations are not Taught.** Grassetti '364 fails to teach at least independent claim 1 and 23 limitations of 1) identifying an individual in need of immune response modulation, and 2) administering to the individual in need of immune response modulation an effective amount of TFD, e.g., as discussed above with regard to alleged anticipation. Further, Grassetti does not teach the many additional specific limitations of pending dependent claims.

The rejection acknowledges that Grassetti '364 does not expressly teach the step of identifying an individual in need of immune response modulation. However, in a failed attempt to state a case, the Examiner mischaracterizes (Action, bottom of page 6) column 12, line 4 of Grassetti '364, as allegedly teaching that "6,6'-dithiodinicotinic acid is taught to maintain or improve the natural defense (immune system) and to keep the cancer cells released during the surgery control." This conclusory statement, central to the rejection, is drawn out of thin air without any factual basis.

At page 5, section 8 of the Action, the Examiner quotes Grassetti '364, column 12, line 4, suggesting "[t]reatment with the regent should be continued for an adequate period of time both before and following surgery until the natural defenses of the organism have destroyed the remaining circulating cancer cells." This statement draws no correlation between the TFDs and destruction of cancer cells. In fact, Grassetti's distinction between

the treatment and the ***natural*** (as opposed to any sort of modulated) defenses makes it clear that, at that time, he assumed that the compound did not affect those defenses. The Examiner is clearly mischaracterizing the statement to assert that it teaches TFDs "maintain or improve the natural defense (immune system) and to keep the cancer cells released during the surgery control." Just because a drug makes a patient feel better during convalescence from a disease does not mean the drug cured the disease or modulated an immune response. For example, an analgesic can make a patient feel better while she has a cold, but unmodulated natural, defenses not the analgesic, ultimately cures the cold.

The context of the specification sheds further light on the meaning of the cited statement. Throughout the Grassetti '364 specification, it is repeatedly made clear that the treatment with TFDs is intended to improve the feeling of well-being. For example, see the Abstract wherein "[c]ancer patients following surgery are treated to lessen pain, induce a feeling of well-being and increase appetite ...". Also see, e.g., column 1, line 27; column 4, line 61; column 4, line 66; column 9, line 22; column 9, line 46; column 10, line 20; column 10, line 52; column 10, line 67; column 11, line 12; column 11, line 23; and column 11, line 34. The "treatment" with the TFDs is well established in the specification solely to provide, e.g., increased appetite and well-being benefits. The natural defenses are not taught as modulated by the TFDs

anywhere in Grassetti '364. In fact, as noted above in the arguments against anticipation, at the time of the filing, it was generally understood that TFDs provided the enhanced feeling of well-being due to their similarity to other mood enhancing compounds. In addition, TFDs were thought to modify the electrical charge on cell surfaces, e.g., to change cell aggregation characteristics. See, e.g., Grassetti, Drugs of the Future 11(7): 559-561, 1986. However, those of skill in the art at the time of filing the present application did not suspect that TFDs functioned in immune response modulation. Therefore, one of skill in the art at the time would not have thought it obvious to provide TFDs to patients in need of immune response modulation.

**The cited combination of references does not provide known elements that function in the same way in the combination as in the references themselves, and the elements are not combined by known methods**. The Examiner, at page 6 of the Action, suggests that Oliver teaches all cancer therapy suppresses immune responses, and Tagawa teaches that modulation of immune responses is one strategy for cancer therapy. Actually, Tagawa teaches modulation by the use of recombinant cytokines with specific immune system cell receptors is one strategy for cancer therapy, but direct injection of the cytokines hazardous and unpredictable, with the cytokines better administered by secretion from recombinant vehicle cells.

Of course, Grassetti teaches oral administration of TFDs to provide a feeling of well-being. The suggested combination requires the Grassetti '364 TFDs to be used to function in a different way in the combination than in the original reference. Therefore, the stated combination can not be considered obvious according to, e.g., *KSR* and MPEP 2143 (A)(2), (B)(2) and ((F)(3).

Oliver only teaches cancer patients can be immune suppressed. Tagawa may teach that immune modulation can generally influence cancer therapy, but does not provide an example of a therapeutic that functions, e.g., non-specifically, without an apparent immune cell receptor, as found in the claims. Critically, Grassetti '364 teaches a therapy which functions to enhance the spirits of a patient, which is different from the function of the claims in modulation of immune responses. The fact remains, no reference teaches TFD functions in immune modulation, so the suggested combination can not possibly teach elements functioning in the same way as in the claims or according to known methods. Therefore, the claims are non-obvious according to *KSR*.

**The suggested combination of elements would have been unpredictable, and one of skill in the art would have not have expected success in providing the claim in light of the references.** Because it was not known that TFDs could influence immune responses prior to the filing of the present application, one of skill in the art at the time would have had no basis to

expect success in modulating immune responses with TFDs. For example, just because the Tagawa cytokine peptides specific to immune cell receptors can be immunomodulatory, one of skill would not predict the different small-molecule TFDs without known cell receptors would have such an activity.

**One of skill in the art at the time would not have been motivated to practice the combination suggested in the rejection.** The Action, at the bottom of page 6, suggests one would have been motivated to identify and administer to an individual in need of immune modulation a TFD "as taught by Grassetti." However, as discussed above, Grassetti '364 does not teach that dithiodinicotinic acid improves natural defense, as erroneously suggested in the Action, and certainly does not teach a beneficial immune modulation. Without a teaching that TFDs modulate the immune system, there is no motivation to employ TFDs in an effort to modulate an immune response.

In summary, Grassetti '364 does not explicitly or inherently teach identifying an individual in need of immune response modulation or administering a TFD to such an individual. The activity of TFDs in immune response modulation is not rendered obvious in light of Tagawa and Oliver's

discussions of cytokines. Therefore, Appellant respectfully requests all the presently pending claims be found allowable.

## CONCLUSION

Appellants submit that the Examiner's rejection of claims 1, 2, 5, 6, 10 to 12, and 20 to 24 is improper. Withdrawal of this rejection by the Examiner or reversal of the rejections by the Board is respectfully requested.

The Commissioner is authorized to charge the fee under 37 C.F.R. §1.17(c) and any other required fees, or to credit any overpayments, to Deposit Account 50-0893.

If a telephone conference would expedite prosecution of the above-identified application, the Examiner is invited to phone the undersigned at (510) 769-3510.

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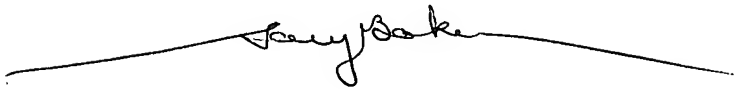
Respectfully submitted,



Gary Baker
Reg. No: 41,595

Attachments:

1) Appendix A – Appealed Claims for 10/044,463;
2) Appendix B - Evidence;
3) Appendix C - Related Proceedings;
4) Notice of Appeal; and,
5) A receipt indication postcard.

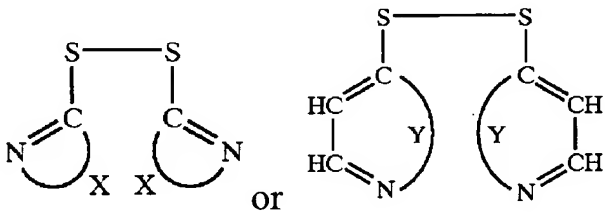
# APPENDIX A

## APPEALED CLAIMS FOR 10/044,463

**1. (Previously presented)** A method for modulating an immune response comprising:

identifying an individual in need of immune response modulation;

administering to the individual in need of immune response modulation an effective amount of a thione-forming disulfide comprising




wherein X and Y represent atoms necessary to form a five-membered or six-membered substituted or unsubstituted heterocyclic ring;

wherein the immune response is selected from the group consisting of: a cellular response, a humoral response and an innate immune response; and,

wherein the individual is other than an individual infected with a retrovirus;

thereby modulating the immune response.

**2. (Original)** The method according to claim **1** wherein the immune response is a cellular immune response.

**3. (Withdrawn)** The method according to claim **2** wherein the cellular immune response is a T cell response and wherein cell populations are increased or lymphoproliferative activity is increased.

**4. (Cancelled)**

**5. (Original)** The method according to claim **1** wherein the immune response is an innate immune response.

**6. (Original)** The method according to claim **5** wherein the innate immune response comprises increasing the natural killer cell population and NK activity.

**7. (Withdrawn)** The method according to claim **1** wherein the immune response is a humoral immune response.

**8. (Withdrawn)** The method according to claim **7** wherein the humoral immune response is a decrease in B cell population or B cell response.

**9. (Withdrawn)** The method according to claim **8** wherein the humoral immune response is an increase or decrease in antibody secretion.

**10. (Original)** The method according to claim **1** wherein the immune response is biased towards a Th1-type response.

**11. (Original)** The method according to claim **10** wherein the Th1-type response is an increased cell population of NK cells or T cells.

**12. (Original)** The method according to claim **10** wherein the Th1-type response is an increased activity in NK cells or T cells.

**13. (Withdrawn)** The method according to claim **1** wherein the immune response is an increase in cytokine levels.

**14. (Withdrawn)** The method according to claim **13** wherein the cytokine is selected from the group consisting of IL-2, IFN-.gamma., IFN-.alpha., IFN-.beta., IL-12, TNF-.alpha., and TNF-.beta..

**15. (Withdrawn)** The method according to claim **1** wherein the immune response is an increase in chemokine levels.

**16. (Withdrawn)** The method according to claim **15** wherein the chemokine is selected from the group consisting of RANTES, IL-8, MIP-1.alpha., MIP-1.beta., MCP-1, lymphotactin, and eotaxin.

**Claims 17 to 19. (Cancelled)**

**20. (Previously presented)** The method according to claim **1** wherein the thione-forming disulfide heterocyclic rings comprise further heteroatoms selected from the group consisting of N, O, and S.

**21. (Previously presented)** The method according to claim **20** wherein the five- or six-membered heterocyclic ring comprises one or more negatively charged substituents.

**22. (Previously presented)** The method according to claim **1** wherein one or both of the heterocyclic rings in the thione-forming disulfide comprises a pyridinyl, pyrimidinyl, thiazolyl, or quinolinyl group.

**23. (Previously presented)** A method of modulating an immune response comprising:

identifying an individual in need of immune response modulation; and,

administering to the individual an effective amount of thione-forming disulfides wherein the compound is selected from the group consisting of: 6,6'-dithiodinicotinic acid (CPDS), 6,6'-dithiodinicotinic acid diethyl ester, 4-carboxypyrimidine-2-disulfide, diethyl 2,2'-dithiobis-(4-thiazol- e carboxylate), and 2,2'-dithiobis-isonicotinic acid;

wherein the individual is other than an individual infected with a retrovirus; and,

wherein the immune response is selected from the group consisting of: a cellular response, a humoral response and an innate immune response.

**24. (Original)** The method according to claim **23** wherein the thione-forming disulfides are administered in a pharmaceutically acceptable carrier.

# APPENDIX B

# EVIDENCE APPENDIX

Tagawa (Current Pharm. Design 6:681, 2000);

Oliver (Cancer Surveys, 13:173-204, 1992); and,

Grassetti, Drugs of the Future 11(7): 559-561, 1986.

# Cytokine Therapy for Cancer

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**Abstract**: Modulation of immune responses by the use of recombinant cytokines or cytokine genes is one of the strategies for cancer therapy. Although host immune responses are complex and many kinds of cells are involved, crucial steps for enhancing anti-tumor responses can be induced by a single or a few cytokines administered. However, cytokines may induce toxic reactions or produce no substantial effects, when the concentration is inappropriate. Administration of recombinant cytokine(s) has advantages in controlling the blood concentration and the biological activity that can be induced by the cytokine. Since cytokines are relatively unstable *in vivo*, cancer patients have to receive a large amount of the recombinant protein to maintain the required blood concentration for biological activity. Administration of the protein is thereby often toxic to the patients. In contrast, secretion of the cytokine from tumor or vehicle cells by gene transfer is another therapeutic maneuver. Previous preclinical studies have shown that cytokines which facilitate type 1 helper T (Th1) cells-mediated immune reactions but not Th2 cells-mediated reactions, when produced in tumors, are effective for anti-tumor responses. Several technical problems to express sufficient amounts of cytokines in appropriate target cells remain unresolved but the potential of cytokine gene therapy is being explored. Cytokine therapy trials also contributes to our present knowledge of how anti-tumor responses can be efectively produced in cancer patients, shedding the light on the generation of tumor-specific immunity in the patients.

## INTRODUCTION

Cytokines possess pleiotropic functions and mediate systemic and local biological actions. Host immune defense system is composed of a complexity of cellular and humoral mechanism and a number of cytokines and chemokines are involved in each step. Identification of these cytokine and chemokine genes has enabled us to dissect the complex reactions and to advance our knowledge on how an immune system is operated. A host defense system is influenced by many kinds of cytokine, and systemic and/or local applications of cytokine molecule(s) to the patients of immunological disorders can be one of the possible therapeutic strategies.

Development of tumors can be in part due to a defect of a host immunosurveillance system and an escape mechanism of tumors from host immune responses may play an important role in the progression of tumors. Suppression of the immune system by immunosuppressive agents can increase the frequency of cancer incidence, and fortification of the host defense mechanism may reduce the incidence. Anti-tumor responses have been gauged in experimental animal models. Based on experimental studies, administration of cytokines can possibly drive an immune system from an anergic state to tumor cells into an activated stage. The role of cytokines in immune responses is not unidirectional. They work for suppression or activation of immune responses under a specific condition. To develop cytokine-mediated therapeutic strategies, we have to understand the roles of cytokines in systemic host defense mechanism. Generation of anti-tumor responses requires multi-step pathways, and many phases of cellular and humoral actions contribute to the establishment of systemic immunity.

Cytokine therapy for cancer is based on the understanding of how an immune system works against tumor cells. To produce effective anti-tumor responses, the mechanism of cytokine-mediated immune responses should be clarified. Although numerous kinds of cytokines operate in an immune system, it is not practical to administer many sets of cytokines or cytokine genes into cancer patients. We have to choose a few cytokines or cytokine genes which can activate an

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immune system appropriately and make the system effective for cancer treatment. A number of preclinical and clinical studies are required to testify the assumption that administration of cytokines or cytokine genes is a potential treatment for cancer patients. In this review, I discuss the cytokines that can be used for cancer treatment, their basic immunological background and their possible clinical use.

## ANTI-TUMOR RESPONSE INDUCED *IN VIVO*

Various kinds of hematopoietic cells are responsible for successful induction of anti-tumor responses. Cytotoxic T lymphocytes (CTLs) are one of the major cell populations, which destroy tumor cells through at least two distinct pathways, a Fas/Fas ligand (FasL) interaction-induced cell death and a specific proteolytic process that is mediated by perforin-granzymes [1]. In the Fas-mediated pathway, FasL on the surface of CTLs binds to its receptor, Fas , which is expressed on target cells, and the cross-linking of the receptors triggers the cascade system toward programmed cell death. The perforin-granzyme pathway is unique to CTLs and these molecules are stored within granules of CTLs. T cell receptor (TCR)-mediated binding of target cells by CTLs stimulates a $Ca^{2+}$-dependent degranulation. Subsequently, a pore-forming agent perforin, and serine proteases granzymes are released into the local environment between the target cells and CTLs. This process generates lysis of target cells. The similar cell-mediated apoptosis occurs in the case of target cell killing that is mediated by natural killer (NK) cells.

To develop CTLs that are specific for a certain type of tumor cells demands several procedures such as presentation of putative tumor antigens and expansion of CTL precursor cells. Engagement of TCR on CTLs is also required for target cell killing. On the other hand, NK cells which belong to a cell population operating in the innate immunity, kill tumor cells whose expression of class I antigens of major histocompatibility complex (MHC) is negative or is significantly reduced [2]. NK cells do not kill class I-positive cells such as normal cells. Therefore, CTLs and NK cells have complementary functions in the cytolytic response. While CTLs recognize target cells that express "unfamiliar" peptide(s) in the context of class I antigens, NK cells look for the cells that are devoid of class I molecules.

Recently, a new cell population of T cells, $NK1.1^+T$ cells, has been identified [3,4]. The population expresses restricted αβ TCR and NK1.1 antigen, a member of the family of NKR-P1 NK cell receptors [5]. The TCR is consisted of invariant α chain, Vα14-Jα281 [6], and polyclonal Vβ8, Vβ7 or Vβ2 chain in mice [7]. The similarly restricted TCR complex, invariant Vα24JαQ and a diverse β chain from a Vβ11 gene segment is observed in human [8]. The restricted TCR recognizes the CD1 antigen, a conserved MHC class I molecule, and a particular sort of glycosylceramides embedded with the CD1 molecule [8,9]. Although the immunological significance of $NK1.1^+T$ cells is not well characterized, this cell population has cytolytic activity toward tumor cells [10,11].

The precise mechanism of how NK cells and $NK1.1^+T$ cells kill tumor cells are not clear yet, but that of CTL-mediated cytotoxic activity has been extensively analyzed. It includes antigen presentation by professional antigen presenting cells (APC), expansion of CTL precursors, recruitment of CTLs into tumors and recognition of tumors. Presentation of putative tumor antigen(s) to helper T cells is an initial step to generate anti-tumor responses. Tumor cell lysis by CTLs is an efferent stage of anti-tumor activity. Two types of T cells recognize different molecular structures, helper T cells bind to a class II molecule with an antigen but CTLs attach to a class I molecule plus an antigen [Fig. (1)]. What kind of antigenic structures is recognized by T cells ? Crystal structures of class I and class II molecules of MHC revealed that a short peptide derived from an antigenic molecule is embedded in a groove of MHC molecules [12,13]. The complex configuration is in fact the target structure recognized by T cells [14]. What kind of molecules can be a tumor antigen or a tumor-associated antigen ? Several types of peptides are clarified in the case of melanoma. Firstly, differentiation antigens that are preferentially expressed in tumor cells but are also found in normal tissues at a certain differentiation stage. This category includes MART-1 and gp100 [15]. Secondly, proteins that are expressed even in normal cells but have a mutation(s) in tumor cells. Thirdly, novel proteins that are generated by alternative transcription or are completely new gene products. At present, several melanoma-associated antigens such as gp100 and tyrosinase are known to be the targets of melanoma-specific CTLs [16], but a few peptides are known as tumor antigens in other human tumors [17].

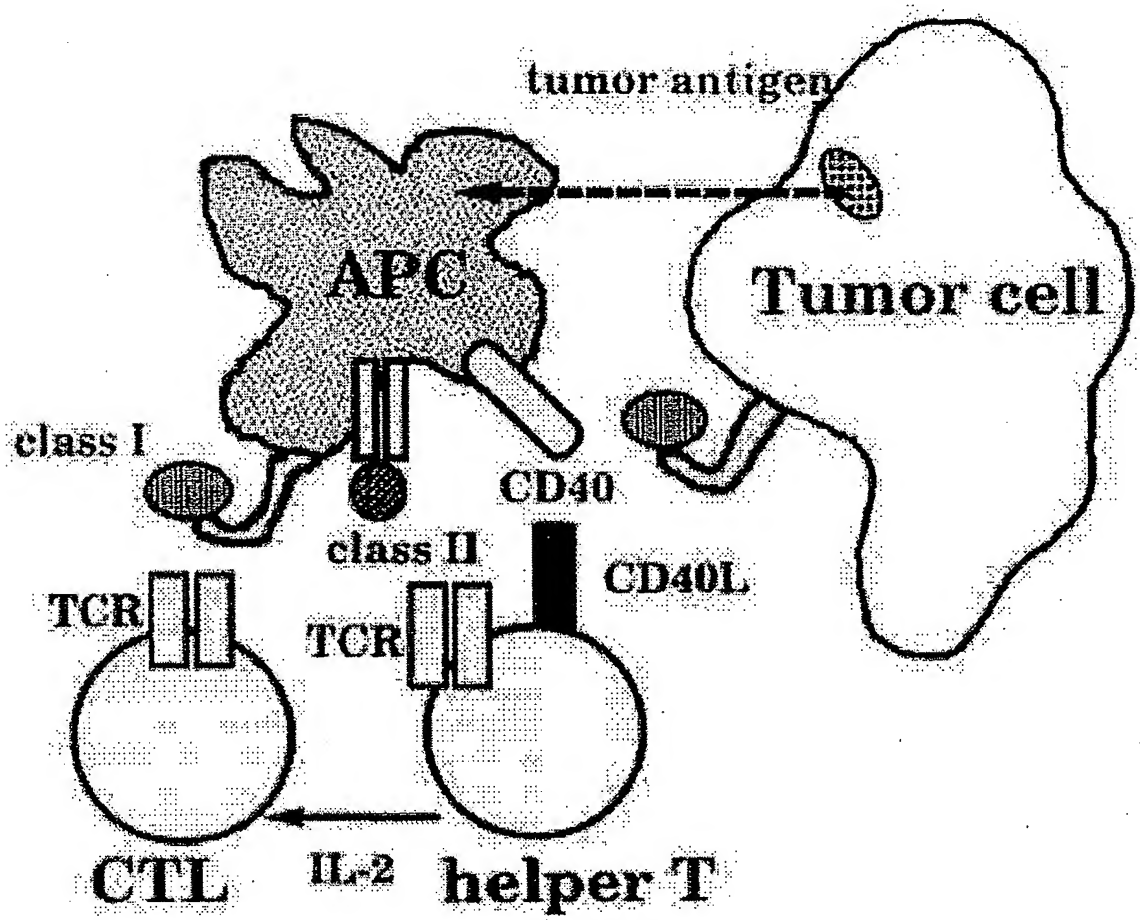


Fig. (1). Interaction between APC and CTLs or helper T cells. Tumor antigen(s) from tumors can be incorporated into APC and a short peptide is presented on cell surface with class II molecules. Helper T cells recognize the complex through TCR and secrete cytokines such as IL-2. APC also present a short peptide that is embedded in class I molecules. CTLs stimulated by the complex undergo proliferation. A signal pathway through CD40/CD40L interaction is important for APC activation.

The way of antigen processing is tightly regulated. Peptides derived from endogenous cytosol proteins are generated by proteasome and are exclusively associated with class I molecules within the lumen of endoplasmic reticulum. They are then transported to cell surface [18]. Instead, exogenous proteins are incorporated into cytoplasm via endosome where limited proteolysis occurs, and antigen peptides are assembled with class II molecules in the late endosome [19]. These mechanisms raise an important problem, because APC have a dual action to present a tumor antigen to helper T cells and to activate CTLs that are specific to the tumor antigen [Fig. (1)]. Since tumor antigen(s) are exogenous to APC, the presentation of the peptide derived from tumor cells should be mediated by the class II pathway. However, APC have to carry the peptides through the class I pathway in order to activate CTLs. Recently, this problem has been solved through the analysis of dendritic cells (DC). DC are professional at antigen presentation and may be the only APC that can stimulate resting or naive T cells, and can consequently initiate CTL-mediated immune responses *in vivo* [20]. Therefore, antigen processing within DC is crucial for anti-tumor responses. Besides antigen presentation by the class II pathway, DC have developed a unique membrane traffic system, endosome-to-cytosol transport [21]. Accordingly, internalized antigens can gain access to the conventional class I pathway. This novel traffic system can confer DC on a specific role in CTL-mediated target cell killing, although DC are heterogeneous in their origin. They are composed of at least two subpopulations, one in the myeloid lineage including Langerhans cells and the other in the lymphoid lineage [22,23].

## Th1 AND Th2 EFFECTOR FUNCTION

Combinatory actions of cytokines can contribute to each cellular and humoral step

toward tumor cell killing, but the other sets of cytokines may counteract the actions, which may induce immune tolerance to tumors. Since a single or a few cytokines can be practically utilized in cytokine therapy, to know how cytokines affect immune responses is important. To activate and endow APC (DC) competent for antigen presentation, stimulation of CD40 molecules on their surface with CD40 ligand (CD40L) that are mainly expressed on T cells is a crucial point [Fig. (1)] [24]. Interaction between costimulatory molecules, CD80 (B7-1) or CD86 (B7-2) expressed on DC, and CD28 on T cells, results in proliferation of T cells and production of inflammatory cytokines such as tumor necrosis factor (TNF)-α [25], which is involved in the upregulated expression of class I and class II molecules. The interaction also increases the secretion of interleukin (IL)-2, interferon (IFN)-α and granulocyte macrophage-colony stimulating factor (GM-CSF). In addition, IL-12 that is also released from DC, contributes to the maturation of T cells.

In the process of expansion of CTLs, they need a help from $CD4^+$ T cells , IL-2 and/or IFN-γ. Maturation of helper T cells is necessary to be competent to produce such cytokines and the function of $CD4^+$ helper T cells is indispensable for the development of CTLs. Based on the pattern of secreted cytokines, $CD4^+$ helper T cells are classified into two distinct subpopulations, type 1 helper T (Th1) and type 2 helper T (Th2) cells [26]. Th1 cells produce IL-2, IFN-γ and TNF-β, whereas Th2 cells secrete IL-3, IL-4, IL-5, IL-10 and TGF-β in general. However, a sensitive assay for cytokine production in individual cells has come to show that many $CD4^+$ T cells cannot easily be grouped into Th1 or Th2 subsets based on the original criteria. At a single cell level, $CD4^+$ T cells exhibit a heterogeneous pattern in secreting various combinations of IL-2, IL-4, IL-10, IFN-γ and TGF-β. Although the T cell subsets may not be categorized by their profile of cytokine production, many experimental studies or naturally occurring immune responses support that the pattern of cytokine production can be linked with Th1 or Th2 dominant immune state. Th1-type immune responses are often associated with inflammation and defense reactions including cytolytic activity, whereas Th2-type responses are antibody-mediated immunity. Correlation of the function of each cytokine with the classified immune state, Th1- or Th2-type response, is not significantly strong, but in general immunological effect of $CD4^+$ T cells can be functionally divided into Th1- or Th2-type. Since Th1-type cytokines promote the maturation and proliferation of CTLs, these cytokines are candidate molecules for therapy in order to enhance cell-mediated immunity. In contrast, Th2-type cytokines rather inhibit inflammatory reactions. Interaction between the two types of responses is reciprocal to each other; Th1-type cytokines are inhibitory to Th2-type responses and vice versa. In that sense, to suppress the secretion of Th2-type cytokines is favorable to the promotion of anti-tumor activity. If the whole immune response is possibly evaluated by relative ratio between Th1-type and Th2-type activity, to shift the immune balance to Th1 dominant state will contribute to the enhancement of anti-tumor responses.

Generation of Th1-type T cells is thereby crucial for cell-mediated immunity. Naive $CD4^+$ T cells can be differentiated into Th1 or Th2 cells, depending on the antigen presented by APC or cytokines concentrated in microenvironment. IL-4 plays a key role in the early phase of this differentiation process and it favors the development of Th2 in the latter phase of the differentiation. In contrast, IL-12 [27] or IL-18 (previously described as IFN-γ inducing factor) [28] can synergistically support the development of Th1 cells. Although IL-12 or IL-18 does not belong to Th1-type cytokines, their biological functions are closely related with Th1-type immunity. They induce the secretion of IFN-γ and augment the proliferation of Th1 and NK cells. These cytokines seem to work synergistically in many facets of Th1 immunity, although the distribution of each receptor is different. Therefore, these Th1-inducing cytokines are also candidate molecules to be used for cancer treatment.

## CYTOKINE THERAPY

What kind of cytokine is effective to induce host immune defense system ? The choice of cytokine(s) for cancer therapy is not easy, because the effectiveness can be dependent on many factors such as the extent of immune suppression in cancer patient and antigenicity of tumor cells. However, it is reasonable to speculate that Th1-type cytokines such as IL-2 and IFN-γ would be favorable to anti-tumor responses, promoting systemic cell-mediated responses. On the other hand, GM-CSF and G-CSF, although they are not directly involved in cytotoxic effect, can circumvent suppressed hematopoiesis that is caused by high-dose chemotherapy and have been evaluated as being clinically beneficial [29]. This

is another category of cytokine-mediated cancer therapy.

Cytokine therapy for cancer treatment was initially tested by the use of recombinant proteins, but the toxic activities accompanied by *in vivo* administration of the proteins became soon apparent and serious adverse actions hampered extensive clinical application of the recombinant proteins. However, subsequent studies have reported that expression of cytokine gene(s) in tumor cells could elicit potent anti-tumor responses. It may be unimportant to discriminate between gene-based therapy and protein-based therapy, because combinatory treatment using both cytokine genes and recombinant proteins, and transduced cell-based therapy are often tried. The advantage of recombinant cytokines is the homogeneity of products and consequently we can measure the biological effects by using standardized amounts of the protein. Recombinant proteins are stable and pure in their quality. However, they are relatively expensive and the half life *in vivo* is unfortunately short in general. In contrast, gene therapy, expression of cytokine gene in tumor cells, is relatively less costly and the advance in vector technology has enabled us to examine the therapeutic effect of *in vivo* gene transfer. However, it is difficult to control the amounts of secreted cytokines and adverse reactions induced by the vector system cannot be avoidable. Nevertheless, the gene therapy using cytokine genes is in fact the most frequently approved trials among gene therapy for cancer. In this review, I firstly describe the cancer therapy using recombinant proteins and then the recent progress in cytokine gene therapy.

## IL-2-BASED IMMUNOTHERAPY

Among cytokines, IL-2 has been widely tested for its anti-tumor activity. However, systemic application of IL-2 induces various adverse effects such as vascular leakage syndrome and marked fluid retention in extravascular space. Hence it cannot be an appropriate procedure, to use high-dose IL-2. A short half-life of IL-2 in human body (less than 10 min) is also a limited factor. Therefore, IL-2 is mainly used ex vivo to expand lymphokine-activated killer (LAK) cells in which T and NK cells are included. Most of the studies using recombinant IL-2 and/or LAK cells showed that the anti-tumor effects by IL-2 or LAK cells alone were not potent enough to suppress the growth of tumors except highly immunogenic tumors.

### Background and Experimental Studies

IL-2 has been originally identified as a mitogenic factor of T cells. Identification of the gene encoding IL-2 molecule showed that it is 15.5 kDa glycoprotein. The receptor of IL-2 was identified to have three subunit molecules, α-, β- and γ-chains. The molecular organization of the receptor, consisting of heterodimeric structure, is strictly related with the binding affinity to IL-2. The biological function of IL-2 is basically to transduce a growth signal to T and NK cells [30]. Although only 10 % of NK cells express high-affinity IL-2 receptors, the remaining 90% can also bind IL-2 [31]. In response to exogenous IL-2, NK cells secrete several cytokines such as IFN-γ, GM-CSF and TNF-α. Resting T cells do not express IL-2 receptors and do not respond to IL-2, unless they receive TCR-mediated signalings. Once T cells are activated by antigens or mitogenic signals, they express IL-2 receptors efficiently and consequently IL-2 enhances the proliferation of activated T cells. This mechanism is advantageous to selective expansion of antigen-primed T cells and eventually contributes to the establishment of immunological T cell-memory.

A variety of therapeutic approaches to treat tumor-bearing mice using recombinant IL-2 has been examined and the studies showed anti-tumor activities by IL-2 to a certain extent [32,33]. To obtain better therapeutic effects, maintenance of *in vivo* IL-2 concentration at a certain level is important, because IL-2 is rapidly cleared from body [34]. While continuous infusion of IL-2 is a choice of therapy, another approach is to activate and expand immunocompetent cells by IL-2 *in vitro* and to transfer them into tumor-bearing mice. When peripheral blood or spleen cells are cultured in the presence of high concentrations of IL-2, a heterogeneous population of T and NK cells are activated to proliferate extensively. These cells, named LAK cells, can destroy a number of tumor cells nonspecifically. The lytic activity of LAK cells are mostly attributable to the activated NK-lineage cells [35,36]. The spectrum of targets by LAK cells is not only tumor cells but virus-infected cells, hapten-modified cells, allogenic cells and even normal lymphocytes [37,38]. The destruction of endothelial cells can be recognized in part as an adverse effect by LAK cell therapy. Preclinical studies have been conducted to test the LAK cell-mediated anti-tumor effect in tumor-bearing animals [39,40]. Administration of LAK cells generated from spleen cells into mice that had tumors and/or metastatic foci could prolong the survival of mice and suppress the expansion of

metastasis. The adoptive immunotherapy has been effective in most of the experimental cases tested and combination of recombinant IL-2 administration and LAK cells infusion produced better effect than either treatment alone [40].

### Clinical Application

Initial application of IL-2 to cancer therapy was explored by systemic administration of natural or recombinant IL-2 into patients of immunogenic tumors such as melanoma and renal cell carcinoma. The observed clinical benefits were not significant, partly because the patients who received IL-2-mediated therapy were in advance stages and to whom standard therapy was ineffective [41]. Combination of systemic administration of IL-2 and LAK cells gave rise to better clinical effects than the therapy with IL-2 alone in melanoma and renal cell carcinoma patients [42]. The overall response rate by IL-2 coupled with LAK cells infusion was reported to be 10-20 % including partial response [42-44]. Thereafter, LAK cells infusion together with chemotherapy and/or administration of cytokines has been tried in various cancer cases. However, clinical outcomes by the strategy turned out to be not impressive [45-47], although better therapeutic effect could be observed to some extent [48]. Nevertheless, migration of lymphocytic cells into primary and metastatic foci after the treatment was noticed and these data suggested that LAK cells could infiltrate into the tumors and work for tumor destruction. To improve the therapeutic effect, tumor-infiltrating lymphocytes (TILs) [49,50] were used. TILs are cells that infiltrate into growing tumors and are obtained from tumor cell suspension. They can proliferate *in vitro* with IL-2 and become predominant in culture after several weeks [50]. Since TILs are considered to be able to migrate into tumor tissues, they may recognize putative tumor antigen(s) or tumor-associated antigen(s). In fact, preclinical studies revealed that TILs could show better therapeutic effect than LAK cells [49]. Most of TILs studied were composed of $CD3^+$ T cells, in contrast to LAK cells. *In vitro* studies also demonstrated that cytotoxic activity of TILs was MHC-restricted, suggesting the presence of tumor antigen(s) expressed on autologous tumors [51]. Migration of infused TILs, after expanded ex vivo, into the targeted tumors was in fact experimentally shown by marking TILs with a radioisotope [52] or the neomycin phosphotransferase gene [53]. Repopulation of TILs to tumors and their lytic action demonstrated the feasibility of TIL-mediated cell therapy. These evidences also indicate a potential use of gene-modified TILs. The majority of the cases who received TIL-mediated immunotherapy in early studies were melanoma and renal cell carcinoma patients, who did not respond to any conventional therapy. Objective regression of tumor foci was observed and the clinical outcome seemed to be promising [54]. However, latter studies showed that significant responses to the therapy were not produced in most cases [55,56].

During the clinical trials, a number of drawbacks associated with IL-2-mediated therapy soon became apparent. The toxicity was in general derived from inflammatory reactions. These toxic reactions were dose-dependent of IL-2 used [57] and belonged to a delayed-type hypersensitivity response. The adverse reactions include fever, malaise, myalgia, nausea and hypotension. These unfavorable effects are not totally due to IL-2 but due to secondary cytokines secreted from activated cells by IL-2, because the onset of toxicity effect was delayed for several hours. NK cells, for example, may produce a number of cytokines such as IFN-γ and GM-CSF, after they are activated. Sudden release of these cytokines consequently activates other cell populations such as monocytes and these cells release proinflammatory cytokines including IL-1, IL-6 and TNF-α. Systemic administration of IL-2 also increase the permeability of blood vessels called vascular leakage syndrome [58]. The phenomenon is not due to hemodynamic changes but in part due to platelet and neutrophil's adhesion to endothelium [59]. Infusion of LAK cells also have similar side effects such as fever and malaise, but most of the patients can tolerate these adverse reactions.

## ANTI-TUMOR EFFECT OF IFN AND TNF-α

### Mechanism of Anti-tumor Effect

Three types of IFN, IFN-α, -β and -γ, have been identified and they have pleiotropic biological activities. The mechanism of IFN-mediated anti-tumor effects is modulation of cell-mediated immunity. In particular, IFN-γ can augment cytotoxic function of CTLs, NK cells and activate monocytes/macrophages [60,61]. Antibody-mediated cytotoxicity is also enhanced. Besides the activation of immunocompetent cells, IFN-γ can augment the expression of class I and II molecules of MHC. The elevated expression of class I antigens on tumor cells increases the antigenicity of tumors, facilitating the recognition

of tumor cells by CTLs [62]. Class II antigens are expressed on APC that can incorporate tumor antigen(s) and their enhanced expression promotes the processing of tumor antigen(s) [Fig. (1)]. Another biological character of IFN-γ is to activate the transcription of other genes involved in an immune response and signal transduction. Among the genes activated, IFN-inducible protein 10 (IP-10) and monokine induced by interferon-γ (Mig) can contribute to anti-tumor activity [63,64]. These chemokines suppress neovascularization of tumor masses and in fact part of anti-tumor effect of IL-12 is mediated by IP-10 and Mig induced [64].

### Clinical Study

Administration of IFN-α caused a good anti-tumor effect to hematological malignancies such as hairy-cell leukemia [65,66], lymphomas [67,68] and chronic myelogenous leukemia [69,70]. IFN-α can inhibit cell growth by inducing G1-arrest but the mechanism of IFN-α-mediated anti-tumor effect remains controversial [71,72]. It has been also used for melanoma and metastatic renal cell carcinoma in combination with other cytokines such as IL-2 [73]. Clinical responses to IFN-α were not clearly observed [74] but the combination therapies using anti-cancer agents and/or IL-2 could improve the clinical outcome. As an adjuvant therapy, IFN-α is being used for hematological malignancy [75-77]. In melanoma cases, high-dose IFN-α was examined for its feasibility in clinical use. Compared with a low-dose of IFN-α, the patients who received high-doses of IFN-α have shown good clinical responses. However, the toxicity generated by high-doses of IFN-α was so strong that only one half of the cases could continue the therapy [78]. The toxicity includes asthenia, neutropenia, and nausea/vomiting [79]. In renal cell carcinoma, remarkable anti-tumor effects were not observed [80,81] but in some cases IFN-α administration can contribute to prolonged survival of the patients [82]. IFN-γ has been also used for advanced cancer patients in combination with IL-2 and TNF-α [83-85]. Although immunological parameters of the patients improved, significant clinical benefits were not seen [84,86].

TNF-α is an inflammatory cytokine and is secreted from many cell types including macrophages and polymorphonuclear cells [87]. It has anti-tumor activity through direct cytotoxic action which is primarily due to apoptosis. TNF-α also promotes intravascular thrombosis within tumor tissues, which leads to necrosis of tumors, and activates immunocompetent cells including neutrophils, macrophages and NK cells [87]. Activation of these cells in turn induce the production of inflammatory cytokine such as IL-1, IL-6 and IL-8 and upregulation of adhesion molecules on cell surface. These secondary reactions can further recruit immunocompetent cells around tumors and consequently enhance tumor destruction.

Administration of TNF-α into tumor-bearing animals prolonged their survival [88]. Clinical trials by combinatory administration of TNF-α and other cytokines were tested [89]. Septic shock and thrombocytopenia were major limiting factors for its clinical application and no apparent anti-tumor effects were observed after systemic administration [90,91], although local application of TNF-α into metastatic foci may be beneficial [92].

## CYTOKINE GENE THERAPY USING Th1-TYPE CYTOKINES

While administration of recombinant cytokine(s) can produce to some extent anti-tumor effects or immunological changes in patients, the toxic reactions preclude the use of large amounts of the recombinant proteins. The other idea is to transfer cytokine gene(s) in tumors and to secrete the cytokine into the vicinity of tumors. The objective of this gene-based therapy is the same as that of protein-based therapy, to activate host immune system with a few kinds of cytokines. Induction of tumor-specific CTLs and generation of potent cytotoxicity are an ultimate purpose of immune therapy. For that purpose, identification of tumor antigen(s) is ideal and tumor-specific CTLs can be induced by immunization with peptide(s) derived from tumor antigens. However, it is often difficult to identify tumor antigens in human cancer. Instead, cytokine gene therapy does not have to reveal such antigen(s). Although immunization with tumor antigens and use of cytokine to enhance immune response against the antigens is preferable, generation of cytokine-mediated immune response is at present the subject to be explored. An advantage of cytokine gene-based therapy is to avoid the toxic reactions induced by systemic administration of cytokine proteins. Transgene-derived cytokines can continuously stimulate an immune system in contrast to recombinant proteins that are easily degraded *in vivo*. However, to measure the local concentration of cytokines in the microenviron-

ment around tumor tissues is often difficult. Moreover, *in vivo* transfer of cytokine gene needs novel vector systems.

The reason why cytokine-secreting tumors can induce systemic anti-tumor immunity is not fully understood. It depends on the cytokines used and experimental models. One of the reasons is that tumor cells may present tumor antigen(s) by themselves and facilitate the activation of helper T cells, when tumor cells happen to express class II molecules. Cytokine secreted from tumors may change the expression of adhesion molecules on endothelium or stroma neighboring tumors and consequently it promotes the recruitment of T cells into the tumor masses. T cells that migrate into tumors can proliferate owing to the cytokine in local environment. Destruction of tumors accompanied by local inflammatory reactions can be facilitated by cytokines and a cascade of a local immune response that leads to systemic immune responses will be generated. Another reason is related to inhibition of neoangiogenesis in tumors. Although the precise mechanism remains ambiguous, a number of experimental models have shown that local secretion of cytokine(s) from tumor cells can induce effective anti-tumor responses [93].

Implantation of cytokine gene-modified tumor cells into syngeneic mice is an initial step to examine the effectiveness of cytokine gene therapy. Using a drug resistance marker and an enzyme-linked immunosorbent assay, cytokine-producing tumor cells can be easily established after gene transfer. *In vitro* proliferation capacity of cytokine-producing tumor cells and the expression levels of MHC class I and class II molecules should be the same as those of parental cells. In order to know which cytokines can induce anti-tumor effects, various cytokine genes and combination of the genes have been examined in animal experiments. One notion is to express Th1-type cytokine genes, which results in enhancing cell-mediated immune responses. We and other showed that Th1-type cytokines, when secreted from tumor cells, can elicit systemic immune responses against cytokine-producing tumors [94-98]. IL-2- or IFN-γ-producing cells were inoculated into syngeneic immunocompetent mice. The representative data using IL-2-producing tumor cells showed that the cytokine-producing tumors, depending on the amounts of secreted cytokine, regressed spontaneously after they had developed small tumors [98,99]. The mice that had rejected cytokine-producing tumor cells developed tumor-specific protective immunity; the mice could rejected untransduced parental tumors that were subsequently inoculated, even when they were administered with tumorigenic cell number; however, they accepted irrelevant syngeneic tumors and the growth of the irrelevant tumors remained the same as that of the irrelevant tumors inoculated in naive mice [98,100]. The observed anti-tumor effect suggests the generation of tumor-specific CTLs in the mice that were inoculated with IL-2-producer cells. In this regard, the induction of CTLs has been shown *in vitro* and *in vivo* [96].

In contrast to Th1-type cytokines, when Th2-type cytokines were engineered to secrete from tumor cells, the rejection of inoculated tumor cells was not observed. In our and other experiments, murine tumors that secrete IL-4, IL-6 or IL-10 developed tumors in syngeneic mice and anti-tumor effects were not evoked [101,102]. Moreover, in the case of IL-10-producing tumors, the tumor size inoculated in syngeneic immunocompetent mice was significantly larger compared with parental tumor size (unpublished data). Since IL-10 is a cytokine that suppresses cell-mediated immune responses [103], local secretion of IL-10 may trigger escape mechanism of tumors from an immune system. In fact, the concentration of IL-10 can be an unfavorable prognostic factor in cancer patients [104,105]. Although we observed that tumor cells secreting Th2-type cytokines were not rejected, other studies reported that Th2-type cytokine-producing tumor cells elicited potent anti-tumor responses, when inoculated in mice [106-109]. The anti-tumor response induced by Th2-type cytokines is attributable partly to the inhibitory action of neoangiogenesis [108] or to nonspecific inflammatory reactions [106]. IL-10 was also reported to inhibit the function of macrophages that may suppress cell-mediated immune responses [110]. Regulation of other molecules such as TGF-β and inducible nitric oxide synthase generated by Th2-type cytokines can contribute to the anti-tumor responses [107,109]. In the case of IL-4-producing tumors, suppressed neovascularization by IL-4 is regarded to be a major reason for anti-tumor activity by the local secretion of IL-4 [111,112]. In spite of these contradictory reports that expression of Th2-type cytokines in tumor cells can induce loss of tumorigenicity, Th2-type cytokine-producers do not in general show anti-tumor effects *in vivo*.

To confirm whether expression of Th1-type cytokines in tumor cells can generate effective anti-tumor responses, we and others tested other

cytokines that are functionally Th1-type-equivalent. IL-15, a novel cytokine which shares some of the IL-2 receptor components (β and γ chain) [113], have similar biological functions as IL-2 has such as support for T cell growth [114,115]. Recently, a human IL-15-specific α subunit gene has been cloned and this subunit is responsible for high-affinity binding of IL-15 [116]. The tissue distribution of IL-15 receptors is wider than that of IL-2 receptors: not only on hematopoietic cells but on endothelial cells [116]. Several toxic reactions such as vascular leakage syndrome that were observed in preclinical and clinical studies using high-dose IL-2 may be due to the activation of IL-15-mediated signaling pathways. The broad distribution of the α subunit can be related with differential biological significance between IL-2 and IL-15. One of the characteristics of IL-15 is that it is a prerequisite factor for development and maturation of NK cells [117,118]. The precise gene regulation of IL-15 is complicated and the amounts of secreted IL-15 also depend on its splicing pattern [119,120].

Anti-tumor effects of IL-15 was demonstrated by expressing IL-15 gene in tumor cells. Inoculation of IL-15-producing tumor cells in syngeneic animals were rejected in syngeneic immunocompetent mice [121,122]. In immunosuppressed nude and severe combined immunodeficient (SCID) mice, the inoculated tumors were not rejected but showed growth retardation, suggesting that the anti-tumor activity was diminished in mature T cell-defective, B cell-defective and NK1.1$^+$T cell-defective conditions [123]. These results showed that not only NK cells but other cells including T cells are important to produce anti-tumor effects. The mice that had rejected IL-15-producing tumors were also resistant to subsequent challenge of lethal dose of parental cells, and this protective immunity was antigen-specific.

It is important to know the cell types that are involved in anti-tumor responses. Inoculation of cytokine-producing tumors into the mice whose specific subpopulation(s) is deleted by administering antibody against the cell type, immunocompromised mice or specific gene-deficient mice by homologous recombination can tell us the cell population(s). What cell type is necessary to generate an anti-tumor response differs among experimental models and cytokines used. In most of IL-2-producing tumors, anti-tumor effects are attributable not only to CD8$^+$ T and CD4$^+$ T cells but to NK cells, granulocytes or macrophages [96,124,125]. These non-T cells are known to express IL-2 receptors. Many reports showed that transduction of IL-2 gene in tumors could effectively induce anti-tumor effects even in nude and SCID mice [124, 126]. Interestingly, tumor-specific protective immunity is induced in nude mice which are deficient in αβ T cells [126]. The protective immunity induced is probably due to γδ T or NK1.1$^+$T cells which are intact in nude mice.

A histological examination of cytokine-producing tumors does not always show the cell types which are responsible for anti-tumor effects. Acquisition of systemic immunity and *in vitro* analysis of immunocompetent mice demonstrated that mature T cells, including αβ and γδ T cells, are involved in anti-tumor activities. However, immunohistochemical staining of cytokine-producing tumors did not clearly reveal the infiltration of T cells into tumor masses. Migration of macrophages is often evident. Macrophage accumulations around the tumors may reflect their scavenger function to digest necrotic tumors. The accumulations can also contribute to antigen presentation of putative tumor antigen(s). Histological findings do not often agree with sequential immunological processes, but tumor necrosis and infiltration of immunocompetent cells are frequently observed in tumors in their regressing phase.

## NOVEL CYTOKINES THAT CAN MEDIATE IFN-γ PRODUCTION

IL-12 and IL-18 belong to a new category of Th1-type-equivalent cytokines [27,28]. These cytokines can induce the production of IFN-γ and promote the differentiation of Th0-type, undifferentiated, immature T cells into Th1-type cells. Both cytokines serve similar biological functions in several aspects, but possess distinctive properties, some of which are derived from the difference of their receptor distribution in tissues. Therapeutic effects by recombinant IL-12 or IL-18 were tested in a number of experimental animal models [127-129]. These results showed that recombinant IL-12 can eliminate established tumors but this dramatic effect cannot be always produced. It depends on the type of tumors. What kind of factors is involved in influencing the effectiveness of IL-12 is not clear. However, several reports showed that migration of T cell into the tumor masses is critical. Secretion of IL-12 often upregulates the expression of adhesion molecules on vascular endothelium such as intracellular adhesion molecule-1 or vascular cell

adhesion molecule [130]. This increased expression of adhesion molecules in a vascular system within tumors and peritumoral area can facilitate the migration of immunocompetent cells. When the infiltration of immunocompetent cells is efficiently induced by IL-12, established tumors are eliminated [130]. However, if the upregulation is not induced, IL-12 fails to show significant anti-tumor effects. Administration of recombinant IL-12 into advanced cancer patients has been examined for its clinical value [131,132], but significant liver damages hampered further clinical trials and gene transfer of IL-12 gene is now being tested.

IL-18 was initially described as a molecule that can induce IFN-γ secretion. The secretion of IL-18 from cells requires a specific enzyme, caspase-1, as found in the case of IL-1β secretion [133,134]. The function of IL-18 is diverse. For example, it enhances NK activity and the expression of IL-2 receptors on T cells, which are similar to the biological activities that IL-12 has. The distinctive functional differences between IL-18 and IL-12 are not clearly understood and most of the experimental studies showed that IL-18 can synergize with IL-12 [135]. However, distribution of respective receptors is different and IL-12 induces and augments the expression of the IL-18 receptor [136]. Since both cytokines induce IFN-γ, several studies reported that the molecules induced by IFN-γ can be a key modulator to determine the effectiveness of the anti-tumor activity by IL-12 or IL-18. The candidate molecules are chemokines such as IP-10 and Mig [64]. These molecules suppress the angiogenesis within tumor masses and work as a chemotactic factor to recruit inflammatory cells around tumors [137]. These chemokines can also enhance antigen-presentation activity of APC [138].

Recently, $NK1.1^+T$ cells were reported to be an indispensable cell population in IL-12-mediated anti-tumor activity. Cui *et al.* showed that $NK1.1^+T$-deficient mice cannot abrogate inoculated tumors with IL-12 but the Vα14-gene transgenic mice that lack the recombination activating gene (thereby have only $NK1.1^+T$ cells) can restore the IL-12-mediated anti-tumor activity [139]. These data does not rule out the involvement of T and NK cells in IL-12-mediated anti-tumor responses, but clearly proves that $NK1.1^+T$ is essential target cells of IL-12.

Expression of IL-12 or IL-18 gene in tumor cells was effective to induce systemic anti-tumor activities [140,141] and these studies also supported the idea that Th1-type cytokine genes are useful in cancer gene therapy. Since IL-12 is heterodimeric (inducible p35 and constitutively expressing p40 subunit), recombinant protein, instead of coexpression of the two genes encoding the subunits, was initially tested. Preclinical experiments demonstrated strong anti-tumor activities. However, IL-12 is relatively toxic and induces severe liver damages as described [131,132]. In gene therapy, both p35 and p40 gene are linked with internal ribosomal entry site and consequently coexpression of both subunits within cells becomes possible [142]. The target cells in which IL-12 may be expressed are not restricted to tumor cells. Recent data showed that IL-12-secreting DC, when injected in the vicinity of tumor cells, are quite effective for tumor eradication [143], because IL-12 secreted from DC plays an important role in antigen presentation particularly through CD40/CD40L pathway [144].

## TUMOR VACCINE USING CYTOKINE-PRODUCING CELLS

Since *in vivo* transduction of cytokine genes into tumor cells has potential problems associated with the use of vectors, tumor cells that were surgically resected and transduced ex vivo were used as a tumor vaccine. Irradiated cytokine-producing tumor cells were injected into naive animals to immunize them and they were challenged with parental tumor cells [145]. A certain dose of irradiation that could eliminate the proliferating capacity of tumor cells but relatively maintained the cytokine production was selected. Irrespective of cell-types, 10-60 Gy-irradiation in general depletes the growth capability but does not seriously damage the cytokine productivity in murine tumors [146]. In some cases, irradiation transiently increased cytokine secretion due to the leakage of cytokines through damaged cell membrane [147]. Immunization with irradiated cytokine-producing tumor cells can generate systemic protective immunity. This vaccine effect is dependent on the immunogenicity of parental tumors. Irradiated parental tumors, when injected into immunocompetent animals, can sometimes generate systemic immunity, and cytokine secretion from the tumors efficiently increases the vaccine effect. This immunization procedure is effective even to established tumors and can decrease the number of preexisting metastatic foci [145, 148]. Expression of GM-CSF gene in tumor cells do not generally induce systemic immunity, when the tumor cells are inoculated. However, when irradiated GM-CSF-transduced tumor cells

are injected, they can elicit potent immunity [149]. The reason why GM-CSF-producing tumors are effective as a tumor vaccine is not clear, but it is probably due to the increased antigen presentation by secreted GM-CSF.

## TARGETTING TUMORS WITH CYTOKINE GENE THERAPY

Efficacy of cytokine gene therapy has been examined in a number of tumor models, including the tumors that are not immunogenic. In the brain, immune responses are not evoked sufficiently since brain is considered to be an immunologically privileged site. One of the reasons is that immunocytes are not able to access brain well because of the blood-brain barrier. Immune responses against intracranial tumors are not as powerful as those against subcutaneous tumors. Furthermore, production of IL-2 from brain tumors can induce brain edema, resulting inefficient anti-tumor effects with cytokine gene therapy [150]. However, an immune response generated by subcutaneous immunization of IL-2-secreting brain tumor cells can induce adequate immunity to the same brain tumors that develops subsequently [151]. The histological examination of the intracranial tumor revealed that microglial responses, which reflect inflammation, and infiltration of macrophages and T cells were observed [152, unpublished data]. These data contradict the previous findings but show that the blood-brain barrier can allow the migration of immunocompetent cells into the brain, when brain tumor develops.

The anti-tumor effects are affected by the administration route of cytokine gene-modified tumor cells. Subcutaneous inoculation of the modified tumors can effectively induce systemic immunity, but administration of the tumor cells into intraperitoneal cavity is less effective. Intravenous injection of the tumors scarcely shows anti-tumor effects. Potency of induced immunity is partly attributable to the degree of antigen presentation. APC such as Langerhans cells are abundant in subcutaneous tissues compared with peritoneal cavity or lung and they may process the tumor antigen more efficiently than the APC in other tissues. Accessibility of APC to the transduced tumors is a key to the effectiveness of cytokine gene therapy.

Is it possible to apply the results of animal studies to human cases? Most of the tested animals were small in size compared with humans, and the most important issue is that human tumors develop spontaneously but animal tumors are experimentally transplanted. Long-term culture *in vitro* often causes the mutations of a number of genes and phenotypic changes in tumor cells. These alterations affect the properties of the tumor cell lines. Modification of the expression levels of major and minor histocompatibility complexes undoubtedly affects the immunogenicity of the tumors. In that sense, all of the cell lines used in preclinical studies are immunogenic in contrast to non-immunogenic tumors developed in patients. Since humans are not inbred animals, genetic variations among patients influence the tumor-host interactions. Accordingly, the difference in class II gene expression among patients and the binding affinity of antigen peptides to the class II molecules affects the efficiency of antigen processing. In other words, a differential immune response of individual patients greatly modifies the clinical outcome, even when the tumors originate in the same tissue and have the same histopathological features.

## CLINICAL APPLICATIONS OF CYTOKINE GENE THERAPY

### Vectors for Gene Transfer

A number of preclinical studies revealed that a certain cytokine gene, when expressed in tumor cells, can produce anti-tumor effects. The efficacy is dependent on many factors such as the antigenicity of tumors and the amounts of cytokine produced. To apply cytokine gene therapy to the treatment of cancer, we have to consider several factors which are critical for successful trials: what kind of cytokine(s) is effective for a particular type of cancer, optimal amounts of secreted cytokine, and how long the production of cytokine should continue. It is also important to evaluate the immunological conditions of patients. Since specific interaction between the patient's immune system and the tumor is difficult to analyze in animal studies, randomized clinical trials are the way to know the efficacy of cytokine gene therapy [153].

There are several technical issues to be settled for the clinical application of cytokine gene therapy; transduction efficiency and safety of vectors. Several methods of gene transfer have been tested. For the standpoint of safety, DNA-conjugated liposome is the best method. Encapsulation of DNA with cationic liposome greatly enhances its incorporation into tumor cells

but further improvement on the transduction efficiency is needed [154]. Retroviral vectors that possess several mutations in the genes in order to avoid unnecessary production of wild-type virus have been frequently used as a vehicle in a number of clinical trials [155]. Since retrovirus infects the cells which are proliferating, preferential integration of the therapeutic gene into tumor cells is an advantage to circumvent the gene expression in non-tumorous tissues. One of the major concerns about retroviral vectors is their potentiality to induce tumorigenesis. Random integration of retrovirus into the chromosome of recipient cells may generate unwanted activation of oncogenes or inactivation of tumor suppressor genes, depending on the integration sites. Although a classical retroviral vector was reported to induce leukemogenesis in the tested primates, there is no incidence reported that the administered retroviral vectors induced tumorigenesis in the patients who received retrovirus-mediated gene therapy.

The major obstacle that retroviral vectors possess is their insufficient transduction rate. Preparation of high titer virus is also technically difficult at present. Moreover, retrovirus is not stable particularly *in vivo*, because retrovirus is susceptible to human complement. Recently, packaging cell lines form human cells are now under development to increase the transduction rate, since the retrovirus prepared from the packaging cells of human origin is resistant to complement-mediated inactivation of retrovirus. Nowadays the usage frequency of retroviral vectors as a clinical-grade vector for cancer gene therapy is becoming lower. Use of retrovirus-producing packaging cells is an alternative choice of vehicle for gene transfer. However, packaging cells derived from murine origin are allogenic and rapidly killed by human immune system. The transduction efficiency is also quite low.

Adenoviral vector has an advantage in high transduction efficiency *in vitro* [156]. However, an immune response to adenovirus, when administered *in vivo*, is induced. The use of adenoviral vectors in human is further complicated by the fact that most of the individuals have immunity to adenovirus. Antibody against adenovirus is further developed in the patients who received adenovirus and CTLs for epitopes present on the viral structural proteins are also generated [157]. It is controversial as to whether these neutralizing antibodies and CTLs can deteriorate the transduction efficiency, because sufficient transduction by adenovirus can be achieved in spite of the presence of the antibody and the CTLs [158]. For most of the cancer gene therapies, transient expression of therapeutic genes is enough to produce potent biological consequences. For that reason, adenovirus is a frequently used vector for cancer gene therapy at present. However, administration of high-dose adenovirus can induce toxic responses in the recipients. In particular, adenovirus preferentially accumulates into the liver. Although systemic administration of adenovirus often induces liver damages [159], high transduction efficiency of adenovirus in the liver may be favorable for treatment of hepatoma. Adverse reactions in the case of local administration of adenovirus is often mild and well tolerable to most of patients.

## Clinical Trials

More than 2200 cancer patients in the world had received gene therapy until September, 1999 and this number is about 70% of total patients who received gene therapy. Scope of targeted tumors is widely distributed and various combinations of therapeutic genes are now being investigated. Among cytokine genes, IL-2 gene is most extensively tried and other cytokine genes frequently examined are IFN-γ, IL-4, GM-CSF and IL-12 [160]. These cytokine genes are used in some cases in combination with the genes encoding costimulatory molecules such as the CD80, which can facilitate antigen recognition process and enhance T cell activation. In preclinical studies, gene transfer of costimulatory molecules increased the susceptibility of gene-modified tumors to host immune system [161]. The other combinatory strategies include simultaneous administration of the gene encoding a putative tumor antigen such as MART-1 in malignant melanomas [162] or an alloantigen such as HLA-B7 [163]. Phase I studies using DNA-conjugated liposome, retrovirus or retrovirus-producing cells showed that the adverse effects caused by gene transfer were minimal and most of the patients are tolerable to the treatment. Limited inflammatory reactions such as pain, swelling and induration at local inoculation sites and transient fever are representative signs and symptoms. Adverse reactions induced by the local administration of adenovirus are similar to those caused by DNA-conjugated liposome and retrovirus. However, intra-arterial administration of adenovirus causes severe liver damages as mentioned. The property that adenovirus tends to infect preferentially hepatocytes, is a benefit to enhance the transduction efficiency of therapeutic

gene(s) into the liver. At present adenoviral vectors are being tested for the treatment of metastatic liver foci via intra-hepatic artery. Adenovirus of higher titer can cause toxic liver damages and in severe cases hemorrhagic tendency is noted. When the liver conditions of the patients are not well maintained, high-dose adenovirus can easily deteriorate the patients' conditions.

Frequency of transgene expression in the patients has been examined. Tissue specimens obtained from the patients are often found to contain the transgene by PCR analysis. Clinical effectiveness of cytokine gene therapy is not fully open to public yet. However, promising results are being reported in several conferences. In melanoma cases, for instance, the recipients developed de novo or increased melanoma-specific, delayed-type hypersensitivity reactions, and $CD4^+$ cell-dominated infiltration around regressing metastases was noticed. None of them exhibited complete or partial regression of main tumors, but some of them experienced a period of disease stabilization, including the shrinkage of a few metastatic foci. A phase II study using liposome-conjugated IL-2 gene showed that more than 50% decrease in the titer of a tumor-associated marker, prostate-specific antigen (PSA), was observed in 38% of the prostate cancer patients, when they received intra-tumoral injection of 300-1500 μg of IL-2 gene [164]. The percentage of the patients whose PSA was decreased more than 25% was over 50%. Since these clinical studies are being examined, the therapeutic potency of cytokine gene therapy is difficult to be evaluated at present. Most of the patients who joined the studies were advanced cases and were refractory to conventional therapies. It also makes the evaluation complicated, because suppression of systemic immune responses is often observed in advanced cases. However, at least in some of the patients, infiltration of immunocompetent cells due to the therapy was detected. This existence of infiltrating cells into the cytokine-producing tumor indicates that immunological responses are actually produced in the patient whose immunity is tolerant to the tumor, although the infiltration may not be directly linked with the clinical outcomes. While the number of responded patients may not be large, these cases are quite important for further analysis on why some patients responded to the therapy and others did not. Investigation on TCR of the infiltrating T cells is a basis to identify the tumor antigens and to develop more sophisticated strategy. Transduction of the TCR genes that are preferentially used in TILs into peripheral blood T cells should potentiate cytolytic activity for tumor elimination [165].

## FUTURE DIRECTION

To produce better therapeutic effects, several strategies is to be investigated. The first tactics is to explore the biological functions of DC that have potent antigen processing activity. These cells are capable of potently activating $CD4^+$ T cells, and releasing cytokines such as IL-2, IL-12 and IFN-γ to promote T cell maturation and proliferation. DC may be the only APC that can present a putative tumor antigen via class I molecules to CTLs. CTLs that can recognize the antigen-MHC complex proliferate with the help of $CD4^+$ helper T cells. Therefore, interactions between DC and $CD4^+$ helper T cells, and/or DC and $CD8^+$ CTLs, are crucial for successful generation of anti-tumor response [Fig. (1)]. What kind of signals is important for the activation of DC and acquisition of antigen processing capacity? DC express CD40 molecule on the surface and stimulation of the CD40-mediated signal pathway enhances the ability of DC to process foreign antigens. Stimulation of CD40 molecule on DC is in general mediated by CD40L that is mainly expressed on $CD4^+$ helper T cells. When DC are activated by CD40L using genetically-modified soluble CD40L or by the transfection of CD40 L gene into DC, anti-tumor activity can be enhanced. The other strategy is to express TNF-α gene in DC. TNF-α activates DC in an autocrine or paracrine manner and promotes the antigen presentation activity. Some of previous studies support this notion [166].

Since DC can be semipurified from bone marrow or peripheral blood cells [20], the source of DC cannot be a limiting factor. However, the morphological characteristics of DC and cell surface markers of DC are not consistent. Consequently, it is difficult to identify the authentic DC lineage at present and to define the biological properties. Future studies should solve these problems. Although population of DC is not identical among the studies, several preclinical studies showed that intra-tumoral injection of DC itself can induce adequate anti-tumor responses. Antigen-pulsed DC are more potent to induce systemic immune responses [167]. Since tumor antigens are not well established except in the case of melanoma, the use of antigen-pulsed DC for clinical studies is restricted at present. However, transduction efficiency of DC with an adenoviral

vector is relative high and DC engineered to load a tumor antigen via adenovirus can possibly be one of the strategies for immune-based gene therapy.

Combination of cytokine genes is another direction. In preclinical studies, coexpression of IL-4 and GM-CSF in tumor cells could induce significant anti-tumor activities [101], although the expression of either cytokine was not effective. The combination seems to promote antigen presentation process and is frequently used to purify and differentiate DC in culture. Suicide gene therapy coupled with the expression of cytokine gene is the other combinatory strategy. Expression of herpes simplex virus-thymidine kinase gene in tumor cells followed by administration of a prodrug, ganciclovir, effectively destroys the tumor masses. Tumor cell death by the suicide gene/prodrug system can stimulate host immune responses [168]. The animals that had eliminated tumors by suicide gene therapy become resistant to parental tumor cells that are subsequently inoculated. Although the molecular mechanism of how tumor cell death produces systemic immunity is not fully understood, several cytokines are known to be involved in the immune response [169]. Therefore, combination of suicide gene therapy and cytokine gene therapy using IL-2 and/or GM-CSF is a possible strategy to enhance the therapeutic efficacy [170].

Cytokine gene therapy has benefits over the therapy using recombinant cytokine proteins. At present, the effectiveness of cytokine gene therapy in clinical trials has not been clearly demonstrated. However, the gene therapy in general does not impair the quality of patient's life in contrast to conventional therapies. One of the directions of immune response-based treatment is to identify tumor antigens and to induce CTLs specific for the antigen peptides as mentioned. The strategy is authentic and straightforward. However, identification of tumor antigens requires the establishment of T cell lines. Moreover, tumor antigens can be different, depending on the class I antigens that encompass the tumor peptides. In melanoma, the peptide antigens harbored in class I molecules are known to be different in individuals according to the heterogeneity of class I antigens. If this is true for other tumors, preparation of a set of tumor antigens for each patient having different MHC is not practical. In contrast, expression of cytokine gene can be achieved in every kind of tumors and can potentially induce CTLs that are specific to the tumors. Cytokine gene therapy thereby circumvents the issue of identifying a specific tumor antigen.

Cytokine gene therapy is a good model to analyze the tolerant immune state found in cancer patients. Tumor cells progressively proliferate in spite of immune responses against them. Tumor cells sometimes secrete immunosuppressive cytokines such as IL-10 or TGF-β. Forced expression of IL-2 gene can overcome the immune suppression and then tumor cells can come to regress. One of the reasons that immune responses do not work against tumors is inhibition of TCR-mediated signalings in T cells. In cancer patients, signal transduction within T cells is often impaired. In particular, ζ chains of the CD3 complex in T cells obtained from tumor-bearing animals are not phosphorylated or even disappeared [171,172]. Dephosphorylation state of the ζ chain cannot transmit the activation signals to the nucleus and secretion of IL-2 from T cells does not occur. This tolerant state is restored by local secretion of IL-2 [172]. Therefore, cytokine gene therapy can break the anergy state induced under tumor-bearing conditions. Conversion of immune tolerance to active immune state can be made in cancer patients by manipulating cytokine gene delivery or administering genetically-modified cells.

## ABBREVIATIONS

| | | |
|---|---|---|
| CTLs | = | Cytotoxic T lymphocytes |
| FasL | = | Fas ligand |
| TCR | = | T cell receptor |
| NK | = | Natural killer |
| MHC | = | Major histocompatibility complex |
| APC | = | Antigen presenting cells |
| DC | = | Dendritic cells |
| CD40L | = | CD40 ligand |
| TNF | = | Tumor necrosis factor |
| IL | = | Interleukin |
| IFN | = | Interferon |
| GM-CSF | = | Granulocyte macrophage-colony stimulating factor |

Th1 = Type 1 helper T

Th2 = Type 2 helper T

LAK = Lymphokine-activated killer

TILs = Tumor-infiltrating lymphocytes

IP-10 = Interferon-inducible protein 10

Mig = Monokine induced by interferon-γ

SCID = Severe combined immunodeficient

PSA = Prostate-specific antigen

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# T Cell Immune Response to Cancer in Humans and Its Relevance for Immunodiagnosis and Therapy

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## INTRODUCTION

The idea that patients' immunity can be harnessed to resist cancer was first considered at the end of the 19th century, when the science of immunity to infectious disease was first established (Currie, 1972). After nearly a century of waxing and waning enthusiasm, there is still considerable uncertainty as to its relevance in the day to day management of cancer patients. Most of the early efforts only helped to clarify the basis of allogeneic transplantation rejection

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**TABLE 1. Cancer in immunosuppressed transplants[a]**

| | Increase | Proportion of cancer (%) |
|---|---|---|
| Kaposi sarcoma | x400 | 5.7 |
| Lymphoma | x90 | 13 |
| Vulva/perineum | x100 | 3 |
| Skin | x29 | 38 |
| Cervix | x14 | 16 |
| Liver | x30 | 10 |
| Breast | na | 10 |
| Prostate | na | 2 |

na = not available
[a]Penn (1988)

(Gorer, 1937), a paradox, as today the principal antigens defined by this process are proving to be the key to understanding tumour rejection (Oliver *et al*, 1989b). Once the genetic basis of the major histocompatibility complex had been worked out in experimental animals (Snell and Higgins, 1951), it was then possible to demonstrate that rejection of syngeneic tumours was also mediated by immune response (Gross, 1943) and that immune memory rested in T lymphocytes (Mitchison, 1953a), as did memory for graft rejection (Mitchison, 1953b). This paper reviews evidence that T cell immune response is relevant to the resistance of cancer in humans, considers laboratory techniques for defining the degree to which the tumour has escaped from immune surveillance and discusses ways in which this observation can be exploited to improve survival in all types of cancer.

## CLINICAL CLUES

### Tumours in Immunosuppressed Individuals

It is a basic tenet of medical science that in order to prove the relevance of a particular process, it is necessary to investigate first the effects of suppression and then the effects of overstimulation of the system. There can be little doubt that suppression of the immune response increases the incidence of some cancers (Table 1) and that the more profound the immunosuppression the more

**TABLE 2. Development of lymphoma after transplantation[a]**

| Method of immunosuppression | Months to onset | Proportion of patients with cancer (%) |
|---|---|---|
| Azothioprine based | 48 | 11 |
| + cyclosporin | 15 | 23 |
| + a – OKT3 | 7 | 64 |

[a]Penn (1988)

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rapid the development (Table 2). However, many of these cancer varieties are not commonly encountered in immunocompetent subjects (Penn, 1988, 1990), and this has been a major reason for the long held scepticism about the general relevance of immune surveillance to cancer development.

Most patients on immunosuppressive drugs will be under constant medical surveillance and will undoubtedly have been encouraged to stop smoking. Moreover, renal transplant recipients, the largest single group of patients to receive immunosuppressive drugs, will have had periods of strict protein restriction while on dialysis. Saturated animal fats are a possible aetiological factor for colon and breast cancer. It is therefore perhaps not surprising that the three most common cancers (breast, colon and lung) may be less frequent in patients on immunosuppressive drugs than some unusual cancers whose cause is not as well understood. At age 35, the average for transplant patients, the expected annual incidence of cancer in the general population is less than 20–30 per 100 000, which is substantially less than the 1 per 200 seen annually during 5 years of follow-up reported in one study of kidney transplant patients (Vogt *et al*, 1990) and substantially more than that seen in patients with renal failure treated with dialysis alone (Port *et al*, 1989).

A possibly more significant indicator of the importance of immune surveillance, although less well documented, is that cancers in immunosuppressed patients are at presentation often more advanced than those in adults with a normal immune response, although not necessarily less differentiated. Supporting evidence for this suggestion comes from the observation that tumours arising in immunosuppressed individuals have less frequent loss of HLA class I and II antigens (List *et al*, 1991). This leads to the paradox that survival may not necessarily be any worse than that with spontaneous tumours. The poor survival of patients with human immunodeficiency virus (HIV) infection in whom lymphomas develop (Roithmann *et al*, 1991) may be partly explained by the increased susceptibility to fulminating AIDS related infections due to immunosuppressive chemotherapy (Roithmann *et al*, 1991). However, testis cancer in HIV infected individuals presents in a more advanced stage than normal both pathologically (Tessler and Catanese, 1987) and clinically (Damstrup *et al*, 1990), but the cure rate is not affected.

## Spontaneous Regression

The second piece of evidence for the existence of immune resistance factors to cancer comes from the many reports of so called "spontaneous" regression of cancer (Table 3) (Challis and Stam, 1990). Although melanoma, kidney tumours and lymphoma predominate, spontaneous regression has been reported for almost every type of tumour. Nevertheless, common cancers such as those of lung, bowel and breast seem less likely to undergo spontaneous regression than do some of the rarer cancers.

Although unexplained "spontaneous" regressions are relatively rare, as Table 4 demonstrates, the more carefully one looks the more frequently one

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**TABLE 3. Spontaneous regression of cancer[a]**

| | No. of cases |
|---|---|
| Leukaemia/lymphoma | 121 |
| Melanoma | 69 |
| Renal cell cancer | 68 |
| Neuroblastoma | 41 |
| GI cancer | 34 |
| Retinoblastoma | 33 |
| Lung and bronchus | 25 |
| Breast | 22 |
| Testis | 16 |
| Others | 75 |
| Total | 504 |

[a]Challis and Stam (1990)

sees it. Before our studies in kidney cancer, it was thought that fewer than 1 in 500 underwent spontaneous regression (Possinger *et al*, 1988). This was certainly so if patients once diagnosed as terminal were just sent home to die. The more often X ray examinations are repeated the higher the frequency of spontaneous regression observed. In our study of kidney cancer, all patients were checked with X rays every month without receiving treatment. Regression was detected in more than 1 in 20 patients (Oliver *et al*, 1989a).

The next section will illustrate a few of these anecdotal observations to demonstrate the spectrum of clinical evidence supporting the concept of tumour resistance mechanisms, although of course not proving that it is the immune system per se that is involved.

The most dramatic example was in a patient who presented with growing lung metastases after nephrectomy (Fig. 1, top) and failed hormone treatment. Over a period of 12 months, the tumours disappeared without any treatment (Fig. 1, bottom). Her husband was an alcoholic, who regularly attacked her. After counselling and support, she remained well for 4 years. However, her husband's alcoholism and violence then recurred and so did her tumour. She left her husband, the new metastasis was excised and she has now remained free of disease for 3 years.

The next two cases illustrate other aspects of the body's resistance to cancer that can only be observed when the cancer can be seen directly. The first case (Fig. 2), however, shows how growing and regressing melanomas can coexist (Bodenham, 1968). This provides a very graphic demonstration of the fact that when the number of cell divisions in a cancer mass is counted and at the same time its rate of growth is measured by serial measurement, it will be apparent that all tumours have a substantial proportion of cells dying. In some patients, 90% of extra cells produced by cell division are lost (Oliver, 1982).

The final case (Fig. 3) shows how cancer can take hold at a site where previous ultraviolet radiation has damaged tissue Langerhans cells (Azizi *et al*,

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**TABLE 4. Comparison of spontaneous regression in testis tumour, melanoma and renal cell cancer**

| | Primary tumour regression in metastatic cases | | Metastases regression (CR + PR) | |
|---|---|---|---|---|
| | presumed complete regression (CR) | apparent "partial" regression (PR) | literature series | author's series |
| Testis seminoma | 8% (52)[a] | 10% (52) | 0.48% (827) | 1/52 |
| Malignant teratoma | 2% (108) | 4% (108) | 0.48% (827) | 0/180 |
| Melanoma | 5.4% (4.344) | 15% (563) | 0.22% (4541) | 1/45 |
| Renal cell carcinoma | na | na | 0.35% (1447) | 5/73 |

For references, see Oliver (1990b)
na = not available
[a]Figures in parentheses are numbers of cases studies

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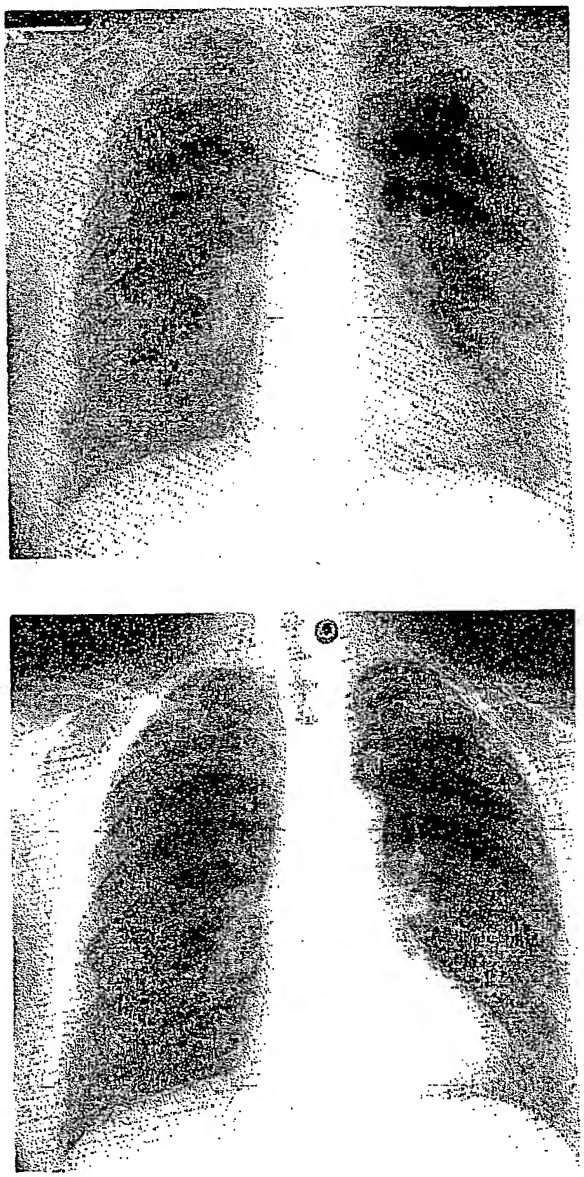

**Fig. 1.** Chest X-ray of patient before (*top*) and after (*bottom*) unexplained "spontaneous" regression

1987), creating an "Achilles heel" in the body enabling tumour cells to escape rejection until there was a breakdown in the patient's resistance mechanisms elsewhere. This patient developed a melanoma at a site where he had had severe sunburn 2 years previously while convalescing from glandular fever. Despite multiple surgery and radiation, his disease kept recurring in this one

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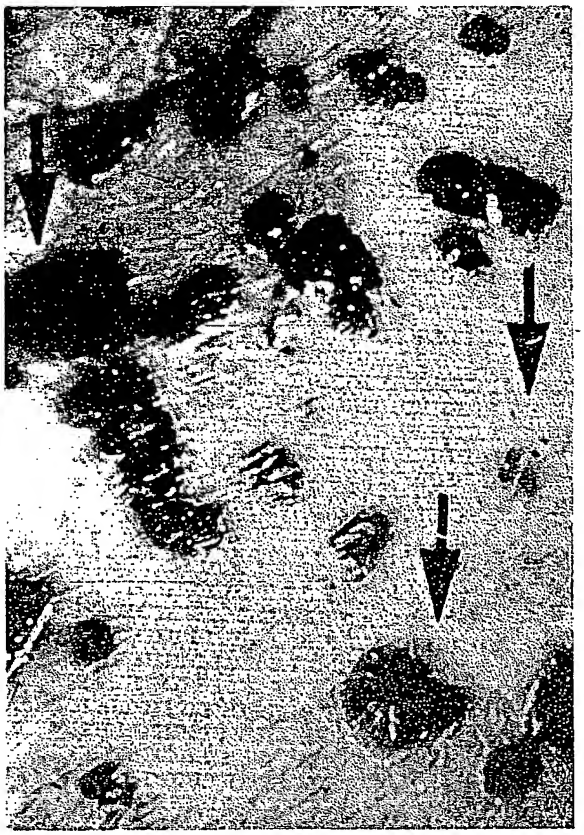

**Fig. 2.** Multiple cutaneous metastases from melanoma showing coincident progressing and regressing lesions

area. Two years before his death, a lesion developed on his right tibia after a football injury, suggesting that melanoma cells may have been circulating in his bloodstream but were eliminated until tissue damage created the environment for cells to settle. No other metastases were discovered until a cerebral haemorrhage, from which he died within 2 hours, was traced to a single brain metastasis.

These anecdotes are not scientific proof for the existence of cancer resistance in humans, but because they come from tumours that are easily visualized sequentially, they give a visual impression of the extremes in the spectrum of tumour behaviour, illustrating the immune surveillance that is probably occurring in a proportion of patients with all types of cancer.

### Tumour Infiltrating Lymphocytes

The third line of evidence for the existence of immune surveillance against cancer comes from data correlating lymphoid cell infiltration with prognosis.

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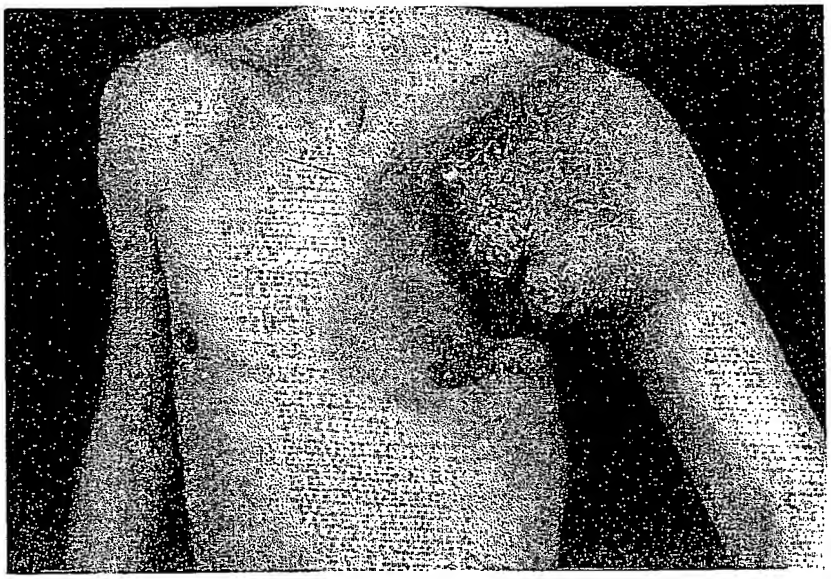

**Fig. 3.** Recurrent melanoma at site of previous major ultraviolet burn

Although first suggested as prognostic factor by MacCarty in 1922, there has been considerable controversy over its significance (Klein *et al*, 1980). As Fig. 4 demonstrates, the action of this prognostic factor is only apparent after 5–10 years of follow-up (Yoshimoto *et al*, 1989), which may explain why some authors have not provided such conclusive evidence for correlation between degree of infiltration and survival. The advent of therapeutic interleukin-2 (IL-2) has aroused considerable interest in tumour infiltrating lymphocytes (TIL), because IL-2 can be used to clone these cells from tumours, expand them in vitro and return them to the patient to induce regression of metastases (Rosenberg *et al*, 1986; Topalian *et al*, 1988). For melanoma (the tumour most thoroughly investigated), about 40% of patients have HLA restricted CD8 positive cytotoxic T cells (Itoh *et al*, 1988). These T cells have been labelled with a neomycin resistance gene and after infusion into the patients were shown to circulate in blood for up to 200 days and were found in excised partially rejected tumours for up to 70 days (Rosenberg *et al*, 1990). The most convincing confirmation that these T cells are specific comes from studies using a polymerase chain reaction based technique to type the melanoma TIL for T cell receptor α-chain usage. These demonstrated restricted oligoclonal representation (Nitta *et al*, 1990; Morita *et al*, 1991).

## IMMUNOLOGICAL ESCAPE MECHANISMS

For successful anti-HLA cell mediated immunity, it is necessary for HLA class II antigens to present antigens to CD4 helper T cells and for class I HLA

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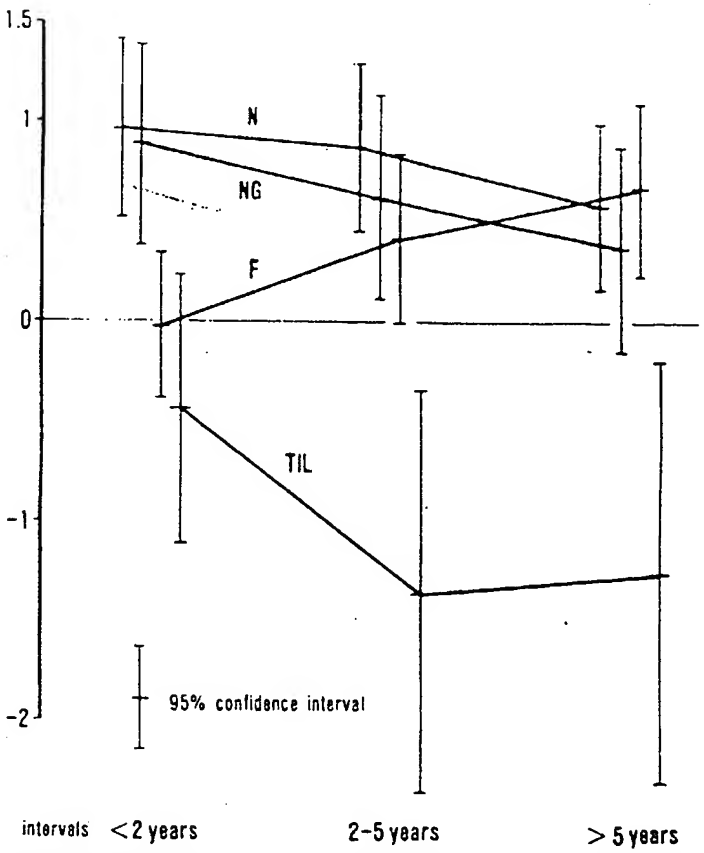


**Fig. 4.** Histopathology prognostic factors regression analysis in breast cancer (n = 480). N = lymph node +, NG = nuclear grade, F = fat invasion, TIL = TIL index. (Yoshimoto *et al*, 1989)

molecules to induce CD8+ cytotoxic T cells. There is increasing evidence that defects of HLA class I and class II antigen expression in tumour cells occur. This may explain the inability of IL-2 to induce class I restricted cytotoxic T lymphocytes (CTL) from TIL in most tumours other than melanoma.

### HLA Class II Antigen Defects in Melanoma and Acute Myeloid Leukaemia

HLA class II antigens are not usually detectable in normal skin, and it has long been known (Natali *et al*, 1987) that class II antigens are switched on more frequently in the more malignant melanomas (Table 5). This is paradoxical, given the observation in autoimmune disease that inappropriate expression of class II antigens may lead to presentation to T lymphocytes of previously non-immunogenetic organ specific antigens and induction of the autoimmune process (Bottazo *et al*, 1983). Alexander *et al* (1989) have provided a possible explanation for this paradox by demonstrating that a melanoma cell grown from a metastasis failed to function, as measured by its ability to present tetanus

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**TABLE 5. Expression of HLA class II on benign and malignant lesions of melanocyte origin[a]**

| | No. tested | No. positive |
|---|---|---|
| Benign | 345 | 21 (6%) |
| Primary | 601 | 297 (49%) |
| Malignant/metastases | 641 | 405 (63%) |

[a]Natali *et al* (1987)

**TABLE 6. Failure of metastatic melanoma DR antigen to present tetanus toxoid to tetanus toxoid immune T cell clone[a]**

| Antigen presenting cell type | Anti-tetanus JFTT-7 | T cell clone CJTT-6 |
|---|---|---|
| Tetanus toxoid (TT) alone | 97[b] | 210 |
| TT + JF non-T cells | 11,607 | 281 |
| TT + CJ non-T cells | 107 | 14,444 |
| TT + JF melanoma (1°) | 25,031 | 164 |
| TT + CJ melanoma (MET) | 107 | 176 |

MET = metastatic
[a]Alexander *et al* (1989)
[b]cpm after 24 hr thymidine incorporation begun 24 hr after exposure to TT

**TABLE 7. Cold target cell inhibition experiments demonstrating specificity of autologous T lymphocyte cytoxicity in AML[a]**

| | Target cell mixtures | | | |
|---|---|---|---|---|
| Patient no. | autologous blasts-$^{51}$Cr ($1 \times 10^4$) | autologous blasts-$^{51}$Cr ($1 \times 10^4$)+ cold autologous blasts ($1 \times 10^4$) | autologous blasts-$^{51}$Cr ($1 \times 10^4$)+ cold autologous remission lymphocytes ($1 \times 10^4$) | autologous blasts-$^{51}$Cr ($1 \times 10^4$)+ cold allogeneic blasts ($1 \times 10^4$) |
| 1 | 34 | 5 | 36 | 31, 34, 37 |
| 2 | 21 | 1 | 18 | (23)[e], 19 |
| 3 | 2 | 0 | 6 | 12, 18 |
| 4 | 43 | 30[b] | 41[b] | 35[b,f] |
| | | 6[c] | 39[c] | 36[c,f] |
| | | 0[d] | 40[d] | 33[d,f] |

Figures indicate specific $^{51}$Cr release caused by in vitro primed remission lymphocytes mixed 20:1 with $^{51}$Cr labelled targets
[a]Modified from Oliver and Lee (1979)
[b]Cold targets $1 \times 10^4$
[c]Cold targets $1 \times 10^4$
[d]Cold targets $4 \times 10^4$
[e]Cold tagets = Daudi
[f]Cold targets - allogeneic phytohaemagglutinin blasts

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toxoid to autologous T cell clones, whereas a similar line from a primary tumour in a patient without metastases functioned as well as autologous B cells in this same assay (Table 6). The defect in presentation could be corrected by transfection into the malignant cell of a normal copy of the defective class II gene (Alexander *et al*, 1991), although it was not possible to determine whether the transfected cell line had altered metastatic capacity.

Most studies of TIL cells from adult solid tumours have failed to generate HLA class I restricted CTL, and possible reasons for this will be discussed in the next three sections. However, one malignancy that has not been studied adequately where HLA class I restricted CTL cells might be present is acute myeloid leukaemia (AML). As with several solid tumours, the level of lymphocyte infiltration in AML patient's peripheral blood at presentation correlates with prognosis (Tupitsyn *et al*, 1990). Furthermore, several studies have demonstrated that AML tumour cells, like melanoma cells, overexpress class II antigens (Lecchi *et al*, 1989), suggesting that they too could have some cells with non-functioning class II antigens (Alexander *et al*, 1989). Early experiments demonstrated that 40% of patients with AML in remission had T cells in the peripheral blood that although not directly cytotoxic for autologous blasts, could become so if the blasts were presented in association with third party allogeneic class II stimulator cells (Lee and Oliver, 1978). In a small series of cold target inhibition studies, it was possible to show that inhibition of cytotoxicity occurred only with autologous and not allogeneic leukaemic blasts or autologous phytohaemagglutinin transformed lymphoblasts (Table 7), suggesting that it might have been class I restricted killing (Oliver and Lee, 1979).

The data from bone marrow transplant studies showing that graft versus leukaemia rejection occurs more frequently after HLA matched allografts than autografts (Kersey *et al*, 1987) and allogeneic leukocyte transfusions (Ford *et al*, 1980) could be in vivo correlates of this in vitro observation. Recently, an attempt has been made to use IL-2 to propagate AML TIL. This was successful in a proportion of patients, particularly those with the M4 type of AML (Table 8), and experiments are in progress to study their cytotoxic potential against autologous leukaemic blasts.

## Thymic Hormones

The thymus atrophies at puberty, and levels of thymic hormones decline from then on (Fabris *et al*, 1984), although the decline is most marked after the age of 60, when the common solid adult cancers begin to reach their peak incidence. Diffusion chamber studies in 1964 first established the critical importance of the thymus as an endocrine organ (Law *et al*, 1964). Subsequent studies in mice demonstrate that thymic hormone can convert broadly autoreactive NK/LAK type lymphocytes from athymic and surgically thymectomized animals into more specific CTL in synergy with IL-2 (Trainin *et al*, 1973; Wagner *et al*, 1980; Mastino *et al*, 1991). There have been no such studies in humans. Since melanoma and AML patients, who provided the best

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**TABLE 8. Influence of acute leukaemic cell subtype on success of lymphocyte expansion[a]**

| Leukaemia 4type | No. of cases | Successful expansion +IL-2 | Successful expansion −IL-2 |
|---|---|---|---|
| ALL | 2 | 1 | 0 |
| AML (M1) | 4 | 0 | 0 |
| AML (M2) | 1 | 1 | 0 |
| AML (M4) | 4 | 3 | 0 |
| AML (M5) | 2 | 2 | 0 |
| AML (EM[b]) | 1 | 1 | 0 |

[a]Nouri *et al* (unpublished)
[b]EM = extramedullary

evidence for specific HLA restricted anti-tumour CTL, were 10–20 years younger than the average patient with adult solid cancer, more studies in this area are clearly indicated. However, recent reports have suggested that use of this active thymic hormone (thymic humoral factor γ-2, an octopeptide) accelerates recovery from persistent virus infection and is associated with increased levels of T cell (Handzel *et al*, 1990).

### HLA Class I Antigen Defects

Intrinsic T cell defects including lack of thymic hormone may partly explain why TIL from adult solid tumours rarely demonstrate major histocompatibility complex (MHC) restricted killing, but a more significant finding is that a substantial proportion of tumour cells from all tumour types studied have variable degrees of reduced expression of HLA class I antigen (reviewed in Oliver *et al*, 1989b). Normal expression is of course a prerequisite for HLA restricted T cell mediated cytotoxicity to occur. Table 9 summarizes our results in bladder cancer. Although global loss of all class I antigen is rare, selective loss of individual polymorphic determinants and generally reduced levels of expression com-

**TABLE 9. Numerical representation of MHC and adhesive molecules on bladder tumours**

| | Normal | Low | Selective loss | Complete loss |
|---|---|---|---|---|
| HLA-ABC | 23/44 (52%) | 144/44 (31%) | 1/18 (5%) | 6/44 (13%) |
| Free heavy chain | 6/41 (15%) | 17/41 (41%) | na na | 18/41 (44%) |
| $\beta_2$m | 26/43 (60%) | 16/43 (38%) | na na | 1/43 (2%) |
| LFA-3 | 26/37 (70%) | 8/37 (22%) | na na | 3/37 (8%) |

na = not applicable

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pared with non-malignant cells are more frequent. Fewer than 20% of patients had normal expression. These studies also demonstrated that it was more difficult to grow TIL from the tumours with decreased class I expression (Nouri *et al*, 1990 and in press).

### Shedding of Tumour and HLA Antigens as an Immune Escape Mechanism

One observation of interest to emerge from the study of HLA antigen loss in lymphomas was the finding that tumours showing HLA loss had higher circulating levels of $\beta_2$-microglobulin (Swan *et al*, 1988), suggesting that the HLA loss seen in these tumours may be due not to switching off of the HLA class I gene but to enhanced turnover and shedding from the cell membrane. This would make it more difficult to detect with standard immunofluorescence techniques, since the incubation time for the assay is sufficient for the cells to cap the antigen-antibody complex off the cell surface. Currie and Alexander (1974) demonstrated that the higher the rate of shedding of tumour antigen from animal tumours the more malignant the tumour behaviour in vivo. Studies in our own laboratory (Lee SK and Oliver RTD, unpublished) have demonstrated that a similar phenomena may be occurring in AML, although there may be an additional factor in that pretreatment but not remission serum contained a paracrine factor that accelerated the rate of shedding (Fig. 5). Recent observations in myeloma demonstrate that exogenously produced IL-6 (possibly from marrow stroma) can accelerate tumour growth (Huber *et al*, 1991) and that in some breast cancer patients, the stroma produce excess amounts of fibroblast growth factors (Winstanley *et al*, 1961) are two further examples of this phenomenon.

### β-hCG, ICAM-1 and Other Tumour Cell Membrane Changes

HLA antigens are not the only cell membrane antigens that are lost during malignant transformation. There has long been interest in altered carbohydrate blood group antigens on bladder cancers (Feizi, 1985) and leukaemias, where decreased expression has been shown to be due to alterations in serum levels of glycosyltransferase enzymes (Kuhns *et al*, 1980). Although these changes may not necessarily affect tumour immunogenicity, they could affect the fluidity of the membrane, leading to alteration of antigen shedding rate.

One other set of antigens that are undoubtedly of importance for effective T cell mediated cytotoxicity are cell adhesion molecules such as LFA-3 and ICAM-1. The importance of these antigens has been well documented in studies of T cell cytoxicity against Epstein-Barr virus infected cells, and several authors have noted that some malignant tumours have such losses, usually, but not always, in association with class I loss. Vanky *et al* (1990) have demonstrated clear evidence that such loss synergizes with HLA class I loss to enhance malignant behaviour (Table 10).

There has long been speculation that an understanding of how the semi-

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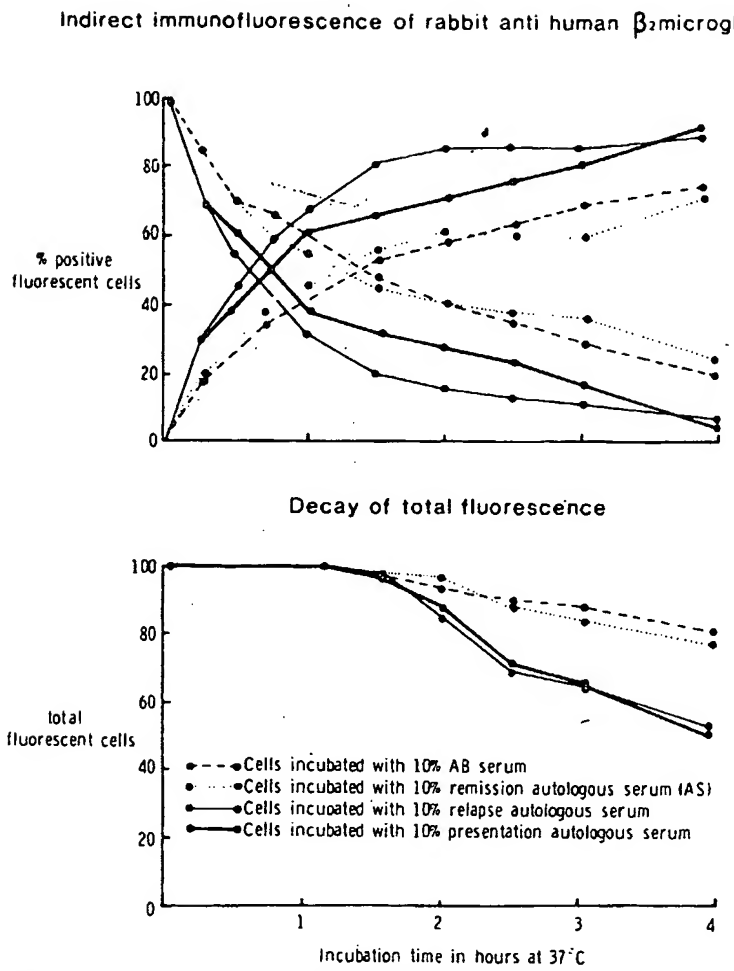


**Fig. 5.** Changes in distribution of $\beta_2$-microglobin in acute myeloid leukaemia blast cells after incubation with remission and relapse autologous serum

**TABLE 10. Effect of HLA class I and ICAM-1 loss on autologous T cell immune response and metastases[a]**

| | Class I+ ICAM 1+ | Only one of them present | Class I- ICAM 1- |
|---|---|---|---|
| Auto-tumour reactivities | 6/7 (86%)[b] | 0/11 | 0/7 |
| Metastatic state | 1/7 (14%) | 6/11 (55%) | 4/7(57%) |

[a]Modified from Vanky *et al* (1990)
[b]Positives/number tested

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allogeneic placenta evades immune rejection might help to explain tumour escape from immune surveillance. Possibly related to this is the accelerated growth sometimes seen in breast tumours that arise during pregnancy compared with similar tumours arising in non-pregnant women (Clark and Chua, 1989).

Further interest in the mechanism of placental escape from immune rejection has arisen from the observation that a minority of tumours from several sites such as bladder, stomach and lung switch on β human chorionic gonadotrophin (β-hCG) in association with accelerated tumour growth, metastasis and possible resistance to treatment (reviewed in Iles *et al*, 1989; Oliver *et al*, 1989b).

In bladder cancer, the β-hCG producing tumours also had reduced HLA class I expression (Oliver *et al*, 1989b; Nouri *et al*, 1990). The World Health Organization has been investigating the use of a vaccine against β-hCG as a contraceptive because it causes the mother to reject the β-hCG bearing trophoblast of the fetus (Stevens and Crystal, 1973). This vaccine might also be considered for the treatment of β-hCG producing bladder cancers.

## APPROACHES TO IMMUNOTHERAPY

### Non-specific Immunotherapy

There is little doubt that superficial bladder tumours (both pTa and pTi) are the human tumours that have shown the most clear-cut evidence of being controlled by stimulation of immune response. Induction of non-specific immune stimulation in the bladder lining by instilling BCG vaccine into the bladder produced complete tumour rejection in more than two-thirds of patients (reviewed in Oliver, in press). Failure of immunological response as measured by a lack of production of IL-2 (Fleishman *et al*, 1989) and failure of induction of HLA class II on tumour cell (Prescott *et al*, 1989) correlated with poor prognosis as measured by tumour response.

### Cytokines, Interleukin-2 and Interferon-α

Interleukin-2 is a cytokine, which acts as a short range messenger regulating the activity of cells involved in immune response (Balkwill, 1989). Interleukin-2 is specifically produced by CD4+ T lymphocytes when they recognize antigen, and it raises lymphocyte concentrations at the site of an active immune response by inducing mitosis in CD4 and CD8 T cells (Taniguchi *et al*, 1986). This cytokine has been under assessment in a phase 1/2 setting but has been seriously evaluated only in kidney cancer and melanoma. There is little doubt that it can produce durable complete remission (Fig. 6 and Table 11). There is a general impression, borne out by study of cumulative series in the literature, that complete remissions are more common and occur in patients with more advanced disease than those seen after interferon-α (IFN-α)

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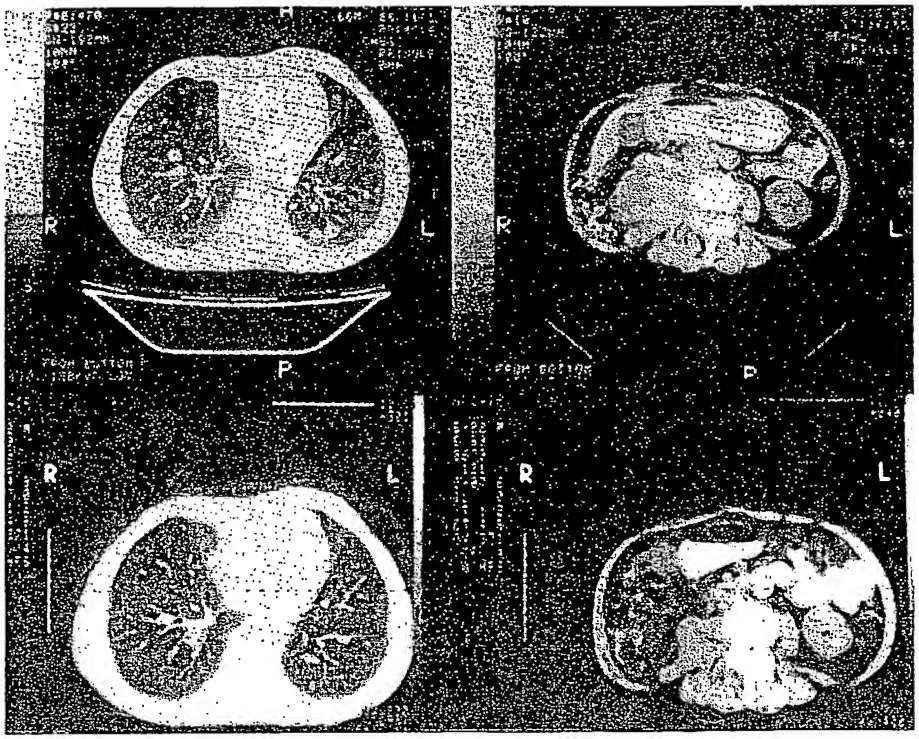


**Fig. 6.** Lung metastases from renal cell cancer before (a) and after (b) IL-2, and recurrence in renal bed of renal cell cancer before (c) and after (d) IL-2

(Tables 12 and 13). The observation that transient autoimmune thyroiditis develops in 14% of patients on interferon, 21% of patients on IL-2 and 40% of patients on combined IL-2 and interferon supports the view that immunological response is relevant to these tumour responses (Pichert *et al*, 1990), and

**TABLE 11. Metastatic renal cell cancer and complete response to BCG, IFN-a, IL-2 and IL-2 plus IFN-a[a]**

| | All cases | | Lung only metastases post-nephrectomy | |
|---|---|---|---|---|
| | no. of cases | CR | no. of cases | CR |
| BCG | 19 | 5% | 7 | 14% |
| IFN-α | 81 | 3% | 17 | 12% |
| IL-2 | 11 | 9% | 4 | 25% |
| IL-2 + IFN-α | 16 | 0% | 3 | — |
| Total | 127 | 3% | 31 | 13% |

CR = complete remission
[a]Oliver RTD, personal observations

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**TABLE 12. Impact of schedule and dose on response of metastatic renal cell carcinoma to IFN-a[a]**

| Frequency of treatment | <3 mu/m² CR+PR | 3-10 mu/m² CR+PR | >10 mu/m² CR+PR |
|---|---|---|---|
| >5/week | 4%+14% (n = 153) | 2%+16% (n = 203) | 2%+13% (n = 494) |
| <5/week | 2%+8% (n = 51) | 0%+13% (n = 54) | 0%+13% (n = 147) |
| <5/wk + q3 V week | | | 3%+22% (n = 207) |

CR = complete remission; PC = partial remission; q3 = repeated every 3 weeks; V = vinblastine
[a]Modified from Horoszewicz and Murphy (1989)

there is some evidence that the development of thyroiditis correlates with duration of response (Atkins *et al*, 1988).

The critical issue for both interferon and IL-2 is the dose schedule, which is increasingly accepted as not following the rules developed for chemotherapy studies. As can be seen from studies of interferon alone, IL-2 alone and interferon and IL-2 in combination (Tables 12 and 13), high dosage causes increased toxicity and shows no clear-cut benefit. However, the interferon data suggest that it may be important to give treatment for a continuous period, which has been routine with IL-2. Evidence from animal IL-2 dose-response studies (Cheever *et al*, 1985) supports the low dose approach, since the area under the curve (more enhanced by subcutaneous injection) may relate better to the biological effect than the peak dose (more marked with intravenous bolus dosage). There is also evidence from in vitro studies that prolongation of stimulus beyond 4 days leads to downregulation of expression of high affinity IL-2 receptors (Gullberg and Smith, 1989). This, taken with the evidence that there may be a bell shaped dose-response curve, ie high dose inhibition of response, in experimental animal models (Talmadge *et al*, 1987), provides ample justification for more extensive exploration of the intermittent lower dose subcutaneous regimens.

Interleukin-2 (a T cell mitogen; Taniguchi *et al*, 1986) and IFN-α (which augments HLA class I expression; Lindahl *et al*, 1974) have differing sites of action on immune response, and there is strong evidence from animal studies

**TABLE 13. Pooled results of IL-2 +/- LAK for renal cancer [a]**

| | Single agent IL-2 | IL-2 + IFN-α | IL-2 + +LAK cells |
|---|---|---|---|
| Low dose IL-2 (outpatient) | 3 + 2/56 (20%) | 4 + 27/145 (21%) | na |
| High dose IL-2 (inpatient) | 15 + 33/328 (13%) | 4 + 17/105 (20%) | 17 + 37/302 (18%) |

na = not available
[a]For references, see Oliver (1991)

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that IL-2 and IFN-α are additive when used in combination (Igo *et al,* 1988; Rosenberg *et al,* 1988). However, to date, the results from clinical trials are conflicting. Although some of the earlier studies showed response rates in excess of 30% (compared with 15% for either agent alone), a recent review of more than 200 patients (Table 13) showed overall responses of 21% (compared with 14% for IL-2 alone and 14% for IFN-α alone). The only formalized randomized trial showed a worse response for the combination treatment than for the single agent treatment (Atkins, 1991). Most of these studies were small pilot studies, and few provided extensive dose-response escalation. The only one to do so showed a bell shaped dose response with lower responses at high doses than at intermediate doses (Rosenberg *et al,* 1989). Perhaps the optimum dosing schedule has yet to be defined. Testing of even lower doses may be needed, since animal studies have shown some evidence that the optimum dose for cytokines in combination is one log lower than the optimum dose of single agents (McHeyzer-Williams, 1989).

Given the current economic climate with controlled access even for heart transplants, which are considerably more successful, the level of activity seen in the renal cell cancer cytokine studies is barely sufficient to justify its routine use in renal cancer, let alone encourage its evaluation in other tumour types. However, if it were possible either by use of clinical criteria (Maladazyz and de Kernian, 1986) or by immunological testing, for example demonstrating that HLA class I antigen expression on tumour was normal, and study of levels of IL-2 (Lissoni *et al,* 1991) or IL-2 receptor in blood (Carteni *et al,* in press) to define subgroups with a high prediction of response, there would be a better case to encourage more widespread study of its use in other cancers. There is already some evidence from the study of renal cell cancer that such a selection maximizes the frequency of response when compared to the frequency of spontaneous regression (Oliver *et al,* 1989a).

### Other Cytokines

Several other cytokines have been investigated in clinical trials, although only two of them, IFN-γ and tumour necrosis factor (TNF), have been examined as thoroughly as IFN-α and IL-2. Neither has demonstrated a major benefit in malignant disease, although IFN-γ has shown considerable promise, leading to a licence for its use in treatment of chronic granulomatous disease (Eckowitz, 1991), and TNF has proven particularly effective at controlling ascites (Raeth *et al,* 1991). It is paradoxical that IFN-γ should prove so ineffective in cancer, given its particularly marked effect in upregulating class II antigens (putatively involved as an intermediary in development of autoimmune disease; Bottazzo *et al,* 1983). Some experimental studies have demonstrated that tumour growth and metastatic spread can be enhanced with IFN-γ (Kelly *et al,* in press). As there is some evidence that there are immune suppressor genes controlled by the class II region (Zaitseva and Brondz, 1990), it is perhaps not surprising that dose may be critical in determining responses.

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## Viral Vaccines and Cancer Prevention

In humans, associations with cancer development have been unequivocally established for three viruses—hepatitis B in hepatoma, Epstein-Barr virus in Burkitt's lymphomas and human papillomavirus (HPV) in cancer of the cervix. These cannot on their own lead to the complete development of terminal cancer, and their principal role is believed to be to provide an initiating proliferative signal that keeps the target tissue dividing faster than normal, thus reducing DNA repair time (Yang *et al,* 1982) and increasing the cells' susceptibility to background mutagenic activity.

Today, the race is on to establish whether by eliminating one of these viruses the associated cancer will disappear. The most likely one to produce information is hepatitis B virus, because a hepatitis B vaccine is now being routinely used in the third world (Whittle *et al,* 1991), where liver cancer is one of the commonest malignancies. However, it is likely to be at least 10–20 years before we will know whether vaccination does protect. Unfortunately, chronic hepatitis B infection develops in about 3% of people vaccinated (Whittle *et al,* 1991).

The majority of bladder cancer patients present with papillary rather than solid tumours. These usually do not show all of the characteristics of malignant tumours but tend to behave more like warts in that they come and go unpredictably and may continue to behave in this way for variable periods, sometimes as long as 10–20 years, before progressing to invasive and metastatic cancer (Oliver, 1990c).

Warts are caused by papillomaviruses (Pfister *et al,* 1986), a group that includes the common verruca virus that most children get these days from swimming in public swimming pools. This group of viruses has been associated with tumours in many animal species. In all of these species, there are many subtypes, and although the associated tumours show a varying degree of malignancy, the majority are benign. The complexity of the relationship of the virus subtype to the degree of malignancy is best illustrated by the classic studies with the Shope papillomavirus in rabbits. These studies demonstrated that it was possible to have benign or malignant tumours from the same virus depending on the genetic resistance of the individual recipient or the amount of carcinogen treatment that was given to individual animals with a benign tumour. These studies have also demonstrated that it was possible to use vaccination to control infection. In humans, the most convincing evidence for involvement of immune response in controlling warts comes from a classic controlled study demonstrating rejection of warts by hypnosis (Spanos *et al,* 1990), presumed to be due to stimulation of the areas of the brain involved in regulation of immune response.

In humans, superficial bladder cancers are as yet not proven to be caused by a specific papillomavirus. However, there have been several anecdotal reports of association with a variety of different types of papillomaviruses (Oliver *et al,* 1989b; Querci della Rovera *et al,* 1989). This is perhaps not surprising, given the variable morphology of these tumours (Oliver, 1990c). There

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is clearly a need to do more detailed human papillomavirus DNA studies in early bladder cancer samples, since a vaccination programme in patients with premalignant lesions would be justified if a papillomavirus antigen common to both cervical and bladder cancer were found.

Evidence has been accumulating that the mumps virus in association with diminished immune response, which allows persistence of chronic mumps virus infection and results in testicular atrophy, may be an initiating factor in testis cancer (Beard *et al*, 1977; Oliver, 1990a,b). Vaccination against mumps virus has now become routine in the UK for all preschool children. At least 25 years' follow-up of more than 5000 children (the lifetime risk of testis cancer is 1:500) would be necessary to prove whether vaccination did protect against the development of testis cancer, although the number would be smaller for individuals with cryptorchidism, who have a 1:50 lifetime risk.

## RELEVANCE OF HOST RESISTANCE FACTORS TO OTHER MODALITIES OF CANCER TREATMENT

There is little doubt that of the two host factors responsible for resistance to cancer, DNA repair enzyme function has a more significant role than immune response. This is because there are few identified adults with DNA repair enzyme defects who have not developed some form of tumour, whereas only a minority of subjects who are immunosuppressed (by treatment with immunosuppressive drugs, infection with an immunosuppressive virus or inheritance of a genetic immunodeficiency syndrome) develop tumours. However, given the greater availability of reagents for reversing immunodeficiency and the unlikelihood that replacement of deficient DNA repair enzymes will undo an established tumour, for the immediate future, exploration of approaches to harness immune resistance seems likely to be more productive.

### Combination of Immunotherapy with Radiotherapy and Surgery

Duration of anaesthesia for surgery (Hattori *et al*, 1980) and radiation, particularly if fractionated over long periods (Sternswald *et al*, 1978), influences the degree of immunosuppression after treatment. Thus, it is surprising that combination of immunotherapy with conventional treatments for all tumour types has been studied very little.

Studies in animals have demonstrated clearly that the degree of immunosuppression correlates with the duration of anaesthesia (Oliver *et al*, in press), and in humans, there is an additional factor, ie blood transfusion, that in renal transplant recipients improves chances of survival of HLA incompatible grafts (Opelz and Terasaki, 1974). By contrast, and providing additional support for the relevance of immune surveillance, whole blood transfusion of cancer patients increases the chance of recurrence of colon, breast and lung cancer patients after radical surgery (Meryman, 1989), whereas one study has demonstrated no such effect from use of packed red cells (March *et al*,

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1990). Further investigation is clearly justified, since use of leukocyte depleted blood is likely to be more cost effective than cytokine treatment.

There have been few large scale formal randomized trials combining immunotherapy and surgery with 5–10 years follow-up, although the results of a trial of BCG/tumour cell vaccine therapy after surgery for colon tumours are perhaps the most convincing evidence for benefit (Hoover *et al*, 1985). The absence of benefit in the subgroup of rectal cancer patients who received prophylactic radiotherapy during the period of vaccination demonstrates that the question of timing may be critical. An additional factor in this study influencing benefit from treatment was the presence of inducible class II antigens on the tumour cells used as vaccine (Ransom *et al*, 1991).

Little work has been done on combining cytokines with surgery, but the data of Ramani *et al* (1986) in experimental animals suggest that there may be a window of opportunity for reducing the frequency of take of tumour cells that escape into the bloodstream during operative manipulation. Pilot studies, done because it was uncertain whether cytokine treatment might delay postoperative healing, have demonstrated no unexpected difficulties when either IFN-α (Cockerell *et al*, 1991) or IL-2 (Oliver RTD, unpublished) is given during the preoperative week.

There has been even less effort to evaluate radiotherapy in combination with immunotherapy, despite evidence of synergy with both interferon and IL-2 in animal studies. The only randomized clinical study involved only 43 patients with squamous cell lung cancer after radical radiotherapy. One year survival was 30% in the 20 who received BCG, compared with 17% in 23 control patients (Pines, 1976).

### Combination of Immunological Treatments with Chemotherapy

Considerable numbers of studies have investigated chemotherapy in combination with immunotherapy, although few have provided evidence of major benefit. The most substantial have been the studies investigating levamisole in combination with 5-flurouracil in colon cancer (Table 14) (Windle *et al*, 1987; Moertel *et al*, 1990). These studies illustrate an important issue of such trials, ie that the benefit of immunotherapy occurs late. This might have been predicted from the data of Yoshimoto *et al* (1989) discussed earlier which demonstrated that TIL cell infiltration was only prognostic for late relapse (see Fig. 4). On the basis of these observations, the US National Institutes of Health consensus conference, summarized in the *Journal of the American Medical Association* (National Institutes of Health 1991), has recommended that this should become standard treatment, which is possibly a little premature given that in the early stage cases, ie stage B, no benefit was demonstrable.

The possibility that there might be more widespread benefit from use of cytokine treatments in combination with chemotherapy comes from the studies of Logothetis *et al* (1991), who demonstrated durable responses with chemotherapy in combination with IFN-α in patients in whom chemotherapy alone had failed.

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**TABLE 14. Impact of 5-FU/levamisole on 2 and 5 year survival of stage C colorectal carcinoma**

| Adjuvant trial | No. of cases | Survival | |
|---|---|---|---|
| | | 2 year | 5 year |
| Moertel *et al* (1990) | | | |
| Observation | 315 | 76% | 52% |
| Levamisole | 310 | 75% | 58% |
| Levamisole/5-FU | 304 | 80% | 75% |
| Windle *et al* (1987) | | | |
| Observation | 49 | 85% | 65% |
| Levamisole | 45 | 80% | 65% |
| Levamisole/5-FU | 47 | 84% | 82% |

5-FU = 5-fluorouracil

## Combination of Biological Treatment with Hormone Therapy

Studies of biological treatment in combination with hormone therapy are even rarer than those of combination with other modalities of cancer treatment. The only positive study (Table 15) was a Japanese study involving only 31 patients (Kosaka *et al*, 1985). However, it identified one issue that may be extremely important for defining the optimum way of identifying synergy between endocrine and immunological treatment. The critical issue was that these authors treated only patients whose tumours had shown a major response to hormone treatment before they were randomized to immunotherapy or control. As hormone sensitivity is a marker of differentiation, this would have been a way of selecting the most differentiated tumours, which the experience with BCG treatment in bladder cancer has demonstrated is critical in achieving response to biological treatment.

A further factor that makes study of hormone and immunotherapy worth further exploration is the fact that castration induces regeneration of the thymus and lymphocytosis (Grossman, 1985). Recent studies in rats have confirmed that this also occurs when synthetic gonadotrophin releasing hormones are used (Fitzpatrick *et al*, 1985). Retrospective analysis of prostatic cancer patients receiving the gonàdotrophin releasing hormone analogue confirmed

**TABLE 15. Influence of immunotherapy (lentinin) on progression free survival of breast cancer responders to hormone therapy[a]**

| | No. of cases | Progression free survival at 3 years[b] |
|---|---|---|
| Controls | 16 | 13% (2) |
| Lentinin | 15 | 46% (7) |

[a]Kosaka *et al* (1985)
[b]$\chi^2$ 4.2, $p < 0.05$

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that lymphocyte levels rise during the first 4 weeks of treatment, and some patients even show reduced numbers of lymphocytes during the surge in testosterone levels that occurs in the first week after treatment is initiated (Joseph J and Oliver RTD, unpublished).

### Immunoprevention of Cancer

Currently, screening techniques for early detection of cervical and breast cancer are being applied widely. Although the approach is relatively economic when assessed on the basis of cost of the test per cancer detected, these figures do not take into consideration the considerable false positive rate, which generates considerable anxiety in healthy individuals, as well as the need for costly reassessments and possible surgical intervention in considerably more individuals than have gained benefit from detection of proven cancer (Mant and Fowler, 1990). Furthermore, the incidence of cancer in the screened population is often higher than in the unscreened population, suggesting that some tumours may be detected by screening that might otherwise have undergone spontaneous regression. Attempts to achieve early diagnosis of prostate cancer by means of annual digital examination (Crawford *et al*, 1991) and ovarian cancer by ultrasound and tumour marker assays (Jacobs *et al*, 1988) have produced equally large numbers of false positives requiring expensive investigations. Many latent foci of prostatic cancer detected at necropsy in patients dying without a diagnosis of cancer (Breslow *et al*, 1977) would also be detected.

Were it possible to develop low cost population based (or high risk group) intervention to boost immune resistance, it is possible that the gain would be more substantial than with any screening programme, since it would not have to be organ specific. In earlier sections, evidence was presented that immune rejection of cancer and precancer is likely to be most efficient with the well differentiated early stages, particularly in the virally induced tumours before immunoselection has led to emergence of clones that have downregulated or mutated HLA class I antigens. Two preliminary studies support this view. In the first study, immunotherapy was given to a randomized group of workers who had a high risk of cancer because they were exposed to high level chemical pollution in a poison gas factory. After a 5 year follow-up, cancer developed in 17 of 146 controls and 7 of 146 individuals treated with nocardia rubra cell wall skeleton (Yamakido *et al*, 1990). The second study, from Korea, demonstrated in a case control study (Table 16) that regular ginseng consumption increased resistance to cancer (Yun and Choi, 1990). Recent animal studies have demonstrated that d-limonene, a component of the oil from the gland in orange peel, may have a similar effect (Elegbede *et al*, 1986).

## CONCLUSIONS

The tumours that develop after immunosuppressive drug treatment provide unequivocal evidence that immune surveillance is relevant to cancer resistance for an increasing proportion of cancer sites, particularly those with evidence

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**TABLE 16. Ginseng consumption and cancer risk[a]**

| Frequency of ginseng intake | Males | | | Females | | |
|---|---|---|---|---|---|---|
| | hospital cancer cases | hospital non-cancer controls | odds ratio (95% CI) | hospital cancer cases | hospital non-cancer cases | odds ratio (95% CI) |
| None | 117 | 56 | 1.00 | 226 | 175 | 1.00 |
| 1–3 times/year | 132 | 108 | 0.58 (0.38–0.90) | 111 | 106 | 0.81 (0.57/1.15) |
| 4–11 times/year | 104 | 115 | 0.43 (0.28–0.67) | 75 | 103 | 0.56 (0.39–0.82) |
| Once/month or more | 83 | 157 | 0.25 (0.16–0.39) | 57 | 85 | 0.52 (0.35–0.78) |
| Total | 436 | 436 | | 469 | 469 | |
| Linear trend test (1 df) | | 45.59 ($p<0.00001$) | | | 3.98 ($p<0.05$) | |
| $\chi^2$ homogeneity test (3 df) | | 47.28 ($p<0.00001$) | | | 16.53 ($p<0.001$) | 4 |

[a]Modified from Yun and Choi (1990)

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for viral involvement in the initiation process. It may well be that this group of tumours will demonstrate the most dramatic benefit from immunotherapy, because the tumours that develop, although they tend to become widespread early, also tend to remain well differentiated and not have reduced HLA class I and II antigens or adhesion molecule expression. These characteristics are increasingly recognized as a means of escape from immune surveillance in cancers arising spontaneously in non-immunosuppressed individuals.

The data that single agent cytokines, interferon and IL-2 produce durable complete remission in a minority of patients with progressive renal cell cancer (predominantly early small volume lung metastases) is the most convincing evidence that manipulation of immune response can produce tumour rejection, although the late follow-up studies from superficial bladder tumours and colon cancer reviewed from the 1970s demonstrate that immunotherapy can indeed improve long term survival advantage for all types of tumours by 10–15%. However, to realize this will require considerable investment of resources in long term studies. Because the beneficial effects of immunostimulation have been more marked in early and premalignant stages of cancer development, there is increasing interest in the idea that low cost population based intervention could yield greater benefit than current cancer screening programmes.

The possibility of using viral vaccines to control tumours with a viral factor in their aetiology is attractive but may prove economically non-viable because of the large number of individuals who will need to be vaccinated without benefit and the long period of follow-up necessary to prove the benefit. The only possible candidates are those viruses that cause additional morbidity other than cancer, as is the case with hepatitis B virus associated with hepatoma and mumps virus associated with testis cancer. However, it will take at least 20 years before any effect of these two vaccines on the incidence of associated tumours will be known.

## SUMMARY

Review of the relationship between the degree of immunosuppression and malignancy in patients on immunosuppressive drugs or immunosuppressed by HIV infection, postoperative blood transfusion or pregnancy provides the most convincing evidence of the importance of intact T cell immunity in resistance to cancer. Defective HLA class I and II antigen expression on tumours arising in non-immunosuppressed individuals and correlation of these changes with increased malignancy and diminished TIL provide the most convincing evidence that one factor necessary to ensure survival of most spontaneous tumours is mutation that enables tumour cells to escape rejection by cytotoxic T cells. These changes are less frequent in tumours in immunosuppressed patients, and preliminary data suggest that use of cytokine therapy is more successful in these tumours and the one in five spontaneous tumours demonstrat-

*Modified from Yun and Choi (1990)

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ing normal expression of HLA antigens and high levels of T cell infiltration. These observations suggest that future use of this therapy should be focused on these cases.

All modalities of cancer therapy except hormone therapy (ie surgery, radiotherapy and chemotherapy) suppress immune responses. Defects of HLA antigen expression are less marked in early cancer. Combinations of immunotherapy with conventional treatment at presentation, including hormone therapy in view of data demonstrating regeneration of the thymus after castration, needs further investigation.

Preliminary results from randomized trials involving nearly 300 individuals accidentally exposed to carcinogens demonstrated nearly 60% reduction of incidence of malignancy at 5 years in the arm receiving non-specific immunotherapy. If confirmed, such an approach might be more cost-effective as an approach for cancer prevention than organ specific cancer screening or vaccination against cancer associated viruses such as hepatitis B or papillomaviruses.

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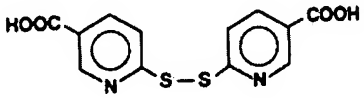
# CPDS

6,6'-Dithiodinicotinic acid
Carboxypyridine disulfide
6,6'-Dithiobis-3-pyridinecarboxylic acid
EN = 113-667
*Antineoplastic*



$C_{12}H_8N_2O_4S_2$ ; Mol wt: 308.33
C 46.75%; H 2.61%; N 9.08%; O 20.75%; S 20.80%

## Synthesis

6-Chloronicotinic acid (I) is reacted with thiourea (II) to give 6-mercaptonicotinic acid (III). This is oxidized to the disulfide with iodine or hydrogen peroxide (1). Scheme 1.

## Description

Off-white powder, m.p. 265°; UV absorption: $\lambda_{max}$ = 251 nm (E = $1.85 \times 10^4$) and 290 nm (E = $1.0 \times 10^4$) in aqueous solution (pH 7.2). When a thiol, e.g. cysteine, is added to a solution of CPDS, 6-mercaptonicotinic acid thione is formed, with $\lambda_{max}$ = 295 nm (E = $1.19 \times 10^4$) and 343 nm (E = $1.0 \times 10^4$) (2).

## History

It was reported in the 1950's that certain carcinostatic alkylating agents act by decreasing the availability of NAD to the cells (3). Based on the premise that NAD is involved in a crucial step of the cancer process, a search was carried out for analogs of nicotinamide which might interfere with this process (interfere with NAD-linked enzymes). It was hoped to achieve therapeutic results in a more selective manner and with less toxicity than the alkylating agents.

CPDS is a result of this search. In 1970, it was found to prevent metastasis in mice (4). Later, it was found to also retard the rate of growth of primary tumors in mice. CPDS has been shown to modify the electrical charge of cell surfaces (5), and thus impair the ability of cells to aggregate (6).

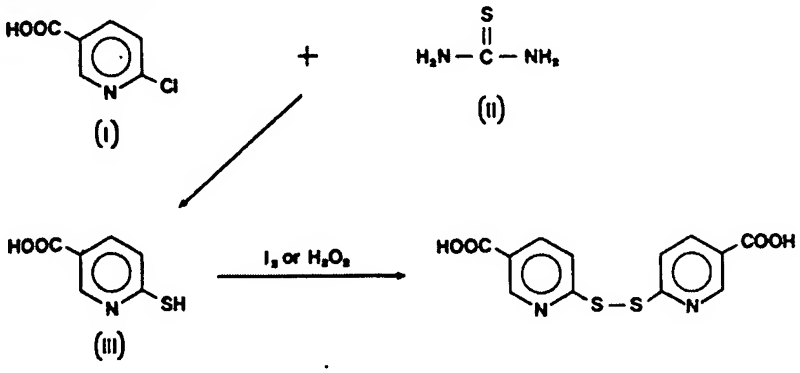
Recent progress in molecular biology has shown that NAD may have a central role in carcinogenesis, as the only precursor of poly(ADP-ribose). Poly(ADP-ribose) synthetase is inhibited by nicotinamide and certain of its analogs. These facts may provide a rationale for the anticancer properties of CPDS.

## Antitumor Activity

When CPDS was administered intraperitoneally to mice bearing intracerebral carcinoma (Ehrlich ascites), the extent of metastasis to the lungs decreased by 75% (4). When CPDS was administered orally (600 mg/kg/day) to mice with transplanted Lewis lung carcinoma in the leg, the number of lung metastases was decreased by 80%, and the rate of growth of the primary leg tumor decreased 50% (7, 8).

No toxicity was observed when CPDS was administered to mice at doses of up to 900 mg/kg intraperitoneally, or for 30 days orally at 900 mg/kg/day. The mice receiving CPDS became more active than those in the control group, indicating the possibility of a stimulating effect. A similar

**SCHEME 1: SYNTHESIS OF CPDS**



stimulating effect was observed in human subjects (9).

Preliminary clinical trials in cancer patients indicate that CPDS administered orally increases survival rate and well-being.

**Pharmacokinetics, Metabolism and Mode of Action**

CPDS reacts with SH compounds to give 6-mercaptonicotinic acid thione (6-MNA) and a mixed disulfide. The latter further reacts with another molecule of the SH compound to give a second mole of 6-MNA and the original thiol (Fig. 1).

It was shown that these reactions occur between CPDS and the SH groups of cell surfaces (Ehrlich ascites tumor, human lymphocytes and platelets) (5). This increase of the negative charges ($COO^-$) around the cell prevents cell aggregation (10) and adhesion (6). This provides a possible mode by which the antimetastatic effect of CPDS takes place: cells treated with CPDS become more negative, and tend to repel each other. This would prevent circulating cancer cells from extravasating and forming metastases.

This mechanism may not be significant in the body upon oral administration of CPDS, since CPDS is rapidly transformed to 6-MNA, which may be the active species in the cell interior. This reaction of CPDS could, however, be of significant aid during surgery: if the operative field is irrigated with a solution of CPDS, iatrogenic spread of the tumor will be prevented by preventing cancer cells from adhering to tissues, and, in addition, any CPDS entering the cells will exert its anticancer activity.

It has been shown (11) by studies with [$^3$H]-labeled CPDS that, when tumor cells are incubated with CPDS, about 70% of the CPDS remains in the cell and cannot be washed off with SH compounds; thus, it is bound in a manner different from a mixed disulfide. This suggests another site of action besides the surface SH groups. Further experiments have shown that both CPDS and 6-MNA are taken up by the cells and become bound to them (12).

Metabolic studies have shown that CPDS, when administered orally, intravenously or intraperitoneally to rats, is rapidly transformed to 6-MNA. UV studies have shown that, when either CPDS or 6-MNA is administered intraperitoneally or intravenously to rats, about 50% of the injected dose is eliminated in the urine within 3 h. Upon oral administration of either CPDS or 6-MNA, the greater part of these compounds appears to remain bound to the tissues (12).

Animal studies have shown that CPDS is equally effective orally or when administered parenterally, dissolved in buffer.

These findings are consistent with the possibility that CPDS and its metabolite 6-MNA, analogs of nicotinamide, exert their anticancer action by inhibiting poly(ADP-ribose) synthetase. Poly(ADP-ribose) is synthesized in the cell from NAD, and it is known that carcinogenesis correlates with a sharp decrease of intracellular NAD and increase of poly(ADP-ribose) (13).

**Clinical Studies**

Two groups of cancer patients have received

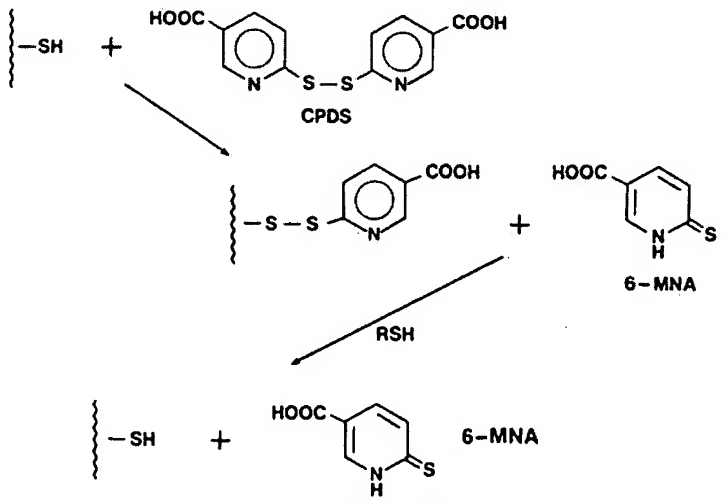



Fig. 1. Metabolism of CPDS.

CPDS (14).

In 12 patients with advanced or terminal cancer CPDS was administered orally at doses of 300-900 mg/day. Most of these patients had received prior treatment: surgery, chemotherapy and/or radiotherapy. CPDS gave them a sense of well-being, and painful and distressing symptoms lessened. Some of the home-ridden patients were able to go back to leading normal and active lives.

Four patients with lung carcinoma were treated surgically by removal of the affected lung. In addition to surgery, the only treatment they received was oral CPDS (600 mg/day), initiated a few days before surgery and continued indefinitely. These patients were in good health after 3, 4, 4 and 6 years, respectively. The life expectancy without treatment is usually about 6 months for this type of tumor.

CPDS, when administered to human subjects, often has a stimulating effect, without any obvious neurological findings or other undesirable side-effects (9).

Oral administration of CPDS causes a decrease of the blood cortisol level. This would be an additional advantage when CPDS is administered to patients with Cushing syndrome (15).

## Source

Ist. Farmacol., Univ. Padova, Largo Meneghetti 2, 35100 Padova (Italy).

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# APPENDIX C

# RELATED PROCEEDINGS

(none)